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Investigating metabolic disease in human induced pluripotent stem cells : adipocyte size, insulin signaling and hepatic lipids

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INVESTIGATING METABOLIC DISEASE IN HUMAN INDUCED PLURIPOTENT STEM CELLS

ADIPOCYTE SIZE, INSULIN SIGNALING AND HEPATIC LIPIDS

Max Friesen

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INVESTIGATING METABOLIC DISEASE IN HUMAN INDUCED PLURIPOTENT STEM CELLS

ADIPOCYTE SIZE, INSULIN SIGNALING AND HEPATIC LIPIDS

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Ter verkrijging van
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geboren te Wessem in 1988

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Summary

Metabolic disease has become pandemic in the developed world, and an incredible burden on the global healthcare system. Given our lack of understanding of its molecular pathology, we are often unable to diagnose patients before they reach an irreversible state of diabetes or cardiovascular disease. Much research has been done on the role of insulin signaling in metabolic disease, as well as the resultant disturbed lipid homeostasis present in cardiovascular disease and atherosclerosis. Here we add to the existing body of work by developing new tools and sketching out the pathology of dysregulated adipose insulin signaling.

Chapter 2 of this thesis discusses lipodystrophy, a lack of adipose tissue, and its effect on metabolism. FPLD2 (Familial Partial Lipodystrophy Type 2) iPSC-derived adipocytes displayed aberrant lipid metabolism, a deficiency in insulin signaling, and perturbed mitochondrial metabolism phenotypes. The FPLD2 adipocytes showed decreased propensity to differentiate into adipocytes, and increased autophagy markers. Altogether these phenotypes hint at the mechanism causing lipodystrophy patients to present with reduced adipose tissue mass.

In chapter 3 we dig deeper into the direct effects of adipose insulin signaling. Through murine adipose-specific deletion of the insulin receptor (*INSR*, CD220) we show dramatic deleterious effects. We observe reduced insulin sensitivity and glucose clearance, as well as disrupted liver metabolism, which led to hepatomegaly and hepatosteatosis and an overall significantly decreased lifespan. We also find AIRKO mice are protected from diet-induced obesity due to the near-complete absence of adipose tissue.

Chapter 4 establishes a relationship between obesity and inflammation. In obese human adipocytes *IRF1* (Interferon Regulatory Factor 1) is upregulated. When ectopically expressed in adipocytes differentiated *in vitro*, *IRF1* alters insulin signaling, and regulates lipid metabolism generating more unilocular and saturated lipid droplets. In mice these adipocytes also attracted more inflammatory macrophages.

In chapter 5 we show the development of a robust differentiation and purification protocol for hepatocytes. We isolate cells based on the mature hepatocyte-specific cell-surface marker ASGR1 (Asialoglycoprotein Receptor 1). These purified cells display increased functional capabilities and were much more similar to human primary hepatocytes. Regardless of iPSC line-to-line variation in hepatocyte differentiation capacity, the purified cells unanimously display a hepatocyte gene expression signature.

Lastly, Chapter 6 utilizes the tool developed in the previous chapter to demonstrate a proof-of-concept study modeling a GWAS-identified SNP *in vitro*. Leveraging a cohort of 68 iPSC lines differentiated into adipocytes and hepatocytes we are able to recapitulate the effect of a SNP on the liver-specific expression of *SORT1* (Sortilin). We replicate the phenotype as found *in vivo* and are able to perform additional studies, such as metabolomics, with the abundance of hepatocyte cellular material provided by the differentiated iPSCs.

The results of all experimental efforts are described in chapter 7, along with a discussion of the implication and future perspectives of the results.

Samenvatting

Stofwisselingsziekte is een epidemie geworden in de ontwikkelde wereld en een belasting voor het mondiale gezondheidszorgsysteem. Gezien ons gebrek aan inzicht in de moleculaire pathologie van stofwisselingsziekte, zijn we niet in staat patiënten te diagnosticeren voordat ze een onomkeerbare toestand van diabetes of hart- en vaatziekten hebben bereikt. Er is veel onderzoek gedaan naar de rol van insuline in metabole ziekten, evenals de resulterende verstoorde lipide-homeostase die verantwoordelijk is voor cardiovasculaire aandoeningen en atherosclerose. Hier voegen we toe aan het bestaande werk door nieuwe hulpmiddelen te ontwikkelen en de pathologie van ontregelde vetcel insulinesignalen te schetsen.

Hoofdstuk 2 bespreekt lipodystrofie, een gebrek aan vetweefsel en het effect ervan op de stofwisseling. FPLD2 iPSC-vetcellen vertoonden een afwijkend lipide-metabolisme, een vermindering aan insuline respons en verstoord mitochondriaal metabolisme. De FPLD2 cellen vertoonden verminderde neiging om te differentiëren tot vetcellen en verhoogde markers van autofagie. Al met al duiden deze fenotypen op het mechanisme waardoor lipodystrofiepatiënten een verminderde hoeveelheid vetweefsel presenteren.

In hoofdstuk 3 gaan we dieper in op de directe effecten van de insuline respons. Door vet-specifieke deletie van de insulinereceptor in muizen tonen we dramatische effecten. We observeren verminderde insulinegevoeligheid en glucose opname, evenals verstoord levermetabolisme, leidend tot hepatomegalie en hepatosteatoze en een significant kortere levensduur. We constateren ook dat AIRKO-muizen worden beschermd tegen obesitas veroorzaakt door dieet, vanwege de bijna volledige afwezigheid van vetweefsel.

Hoofdstuk 4 legt een verband tussen obesitas en ontsteking. In obese menselijke vetcellen is *IRF1* verhoogd. Wanneer het tot expressie wordt gebracht in *in vitro* gedifferentieerde vetcellen, veranderde *IRF1* de insuline respons en het lipidenmetabolisme, waardoor meer unilaterale en verzadigde vet-druppels werden gevormd. In muizen trokken deze vetcellen ook meer inflammatoire macrofagen aan.

In hoofdstuk 5 ontwikkelen we een robuust differentiatie- en zuiveringsprotocol voor hepatocyten. We isoleren cellen op basis van de volwassen hepatocyten-specifieke marker *ASGR1*. Deze cellen vertoonden verhoogde functionaliteit en waren meer vergelijkbaar met menselijke primaire hepatocyten. Ongeacht variatie tussen iPSC lijnen in de differentiatiecapaciteit, vertonen gezuiverde cellen een hepatocyte-genexpressiesignatuur.

Ten slotte gebruikt hoofdstuk 6 het protocol uit hoofdstuk 5 om een SNP geïdentificeerd in een GWAS *in vitro* te bestuderen. Door gebruik te maken van een cohort van 68 iPSC-lijnen gedifferentieerd tot vetcellen en hepatocyten kunnen we het effect van deze SNP op de lever-specifieke expressie van *SORT1* recapituleren. We repliceren het fenotype zoals dat *in vivo* wordt gevonden en zijn in staat aanvullende mechanistische onderzoeken uit te voeren, zoals metabolomics, met de overvloed aan cellulair materiaal van hepatocyten die mogelijk is met de gedifferentieerde iPSC's.

De resultaten van alle experimentele inspanningen worden beschreven in hoofdstuk 7, samen met een bespreking van de implicaties en toekomstperspectieven van de resultaten.

Chapter 1: Introduction

Inappropriate cellular response to insulin is an affliction called insulin resistance (IR). Insulin signaling maintains metabolic homeostasis through mechanisms such as blood glucose clearance, regulating lipid homeostasis, and effects on appetite. IR leads to hyperglycemia due to decreased glucose uptake in peripheral tissues, most notably the muscle, liver and fat. Hyperglycemia, along with other mechanisms, causes IR to pose an increased risk for Type II Diabetes (T2D) and cardiovascular disease (CVD) (Rask-Madsen and Kahn, 2012). Obesity often accompanies IR, and its incidence has doubled over the last 30 years as shown in Figure 1. The disease group of IR, T2D, CVD and obesity together has often been called metabolic disease or metabolic syndrome (Alberti et al., 2005).

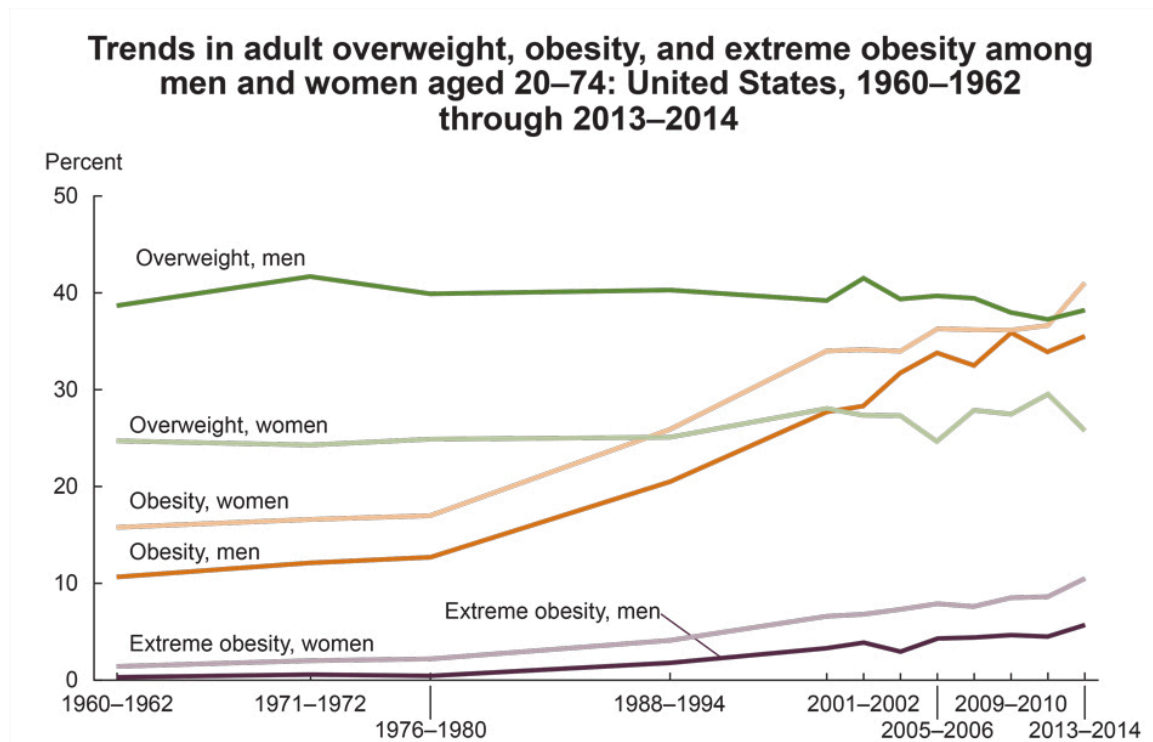


Figure 1. Age-adjusted obesity rates for people in the United States. Age groups are 20–39, 40–59, 60–74. Overweight is BMI >25kg/m², obesity is BMI >30kg/m², extreme obesity is BMI >40kg/m². Source: NIH NIDDK.

The etiology of IR is incredibly complex, due to the interplay of genetics and environment. Most genetic contribution to IR is polygenic, with each variant contributing only a minor amount to the overall pathology. On top of this multigenic landscape there is a strong contribution of environmental factors, including diet, exercise and lifestyle. Here we focus on the genetics and molecular mechanisms of insulin resistance and related metabolic diseases.

Metabolic homeostasis

Metabolism comprises the chemical reactions by which the body converts nutrients into energy to maintain life. It encompasses a vast amount of processes meant to break down substances to yield energy, catabolism, or to synthesize the building blocks necessary for life, anabolism. One of the premier players in metabolism is glucose, the first-choice energy-generating molecule for most cells in the body, and the energy-carrying unit into which most dietary macronutrients are converted. The main organs involved in postprandial glucose uptake are skeletal muscle, the liver and adipose tissue (Petersen et al., 2017). Adipose tissue and the liver also subsequently store glucose by utilizing it to further synthesize lipids (Ameer et al., 2014). Through this process long-term energy storage can be achieved by accumulating lipid reserves. This regulates and impacts whole-body metabolic homeostasis and energy levels. Though both organs generate triglycerides out of glucose, adipocytes have the specialized function of storing large fat reserves for extended periods of time. This process is closely regulated by a number of hormones, most notably glucagon and insulin, which affect both glucose and lipid metabolism (Cherrington and Vranic, 1971). To regulate the body's intake of nutrients, these hormones affect a hypothalamic circuit. Through the same brain axis ghrelin and leptin affect hunger, satiety and appetite (Massadi et al., 2017). In a healthy body under hypoglycemic conditions, liver glycogen and fat triglycerides are broken down into glucose and free fatty acids and released into the bloodstream for energy use by other organs (Nguyen et al., 2008). However, improper glucose control can give rise to many conditions. For example, hypoglycemia can lead to heart palpitations, shakiness, confusion, seizures and loss of consciousness, which can be terminal if untreated (Cryer, 1999). Hyperglycemia on the other hand, is one of the hallmarks of diabetes, and extended exposure to high blood glucose levels leads to cardiovascular complications, diabetic nephropathy, retinopathy and cataracts (Ruderman et al., 1992). It has also been shown that hyperglycemia leads to microvascular complications, which most likely precedes and contributes to ensuing cardiovascular disease. This is hypothesized to be because of superoxide accumulation (Brownlee, 2001). The increase of reactive oxygen species occurs due to cells attempting to combat the hyperglycemia by overstressing their mitochondria, which induces mitochondrial dysfunction and endoplasmic reticulum stress. This promotes cellular damage and in turn potentiating development as well as progression of diabetic complications.

Lipodystrophy

Lipodystrophy is defined as a medical condition in which the body is unable to maintain appropriate adipose tissue (Hegele et al., 2007). Lipodystrophy is a disease group that can be divided into congenital and acquired lipodystrophies, with multiple types of each (Decaudain et al., 2009; Leiter and Semple, 2017). Here we focus on one type of congenital lipodystrophy, namely Familial Partial Lipodystrophy Type 2 (FPLD2), also called the Dunnigan Variety (Hegele et al., 2007). It is a rare genetic metabolic condition characterized by a loss and abnormal distribution of subcutaneous adipose tissue. In this type specifically, there is an

atrophy of trunk and limb subcutaneous tissue, while there is excess supraclavicular fat. This lack of adipose causes insulin resistance, driving progression to T2D as well as fatty liver and hyperlipidemia followed by atherosclerosis. It also leads to reduced serum concentrations of adiponectin and leptin, further exacerbating these deleterious metabolic effects.

FPLD2 is a monogenic disease caused by mutations in *LMNA*, with the most common genetic variation being the missense mutation R482W (Capanni, 2005; Decaudoine et al., 2009). Mutations in *LMNA* can lead to a wide range of syndromes called laminopathies, which includes premature aging syndromes, myopathies and neuropathies (Worman and Bonne, 2007). The R482W mutation displays no association with laminopathies outside of lipodystrophy. The mutation is known to be in the C-terminal tail of the protein where it does not alter three-dimensional structure, but its mechanism of causing lipodystrophy is unknown (Krimm et al., 2002). It is possible this mutation causes structural defects in the nuclear envelope and disrupts protein-protein interactions that are important for adipogenesis.

Insulin signaling

Insulin is the most critical hormone for energy homeostasis, as it regulates both glucose and lipid metabolism. Upon high blood glucose levels, such as after a meal, pancreatic beta cells secrete insulin into the bloodstream (Goren, 2005). When it reaches its target tissues, it promotes glucose and fatty acid uptake, as well as glycogen and triglyceride synthesis (Saltiel and Kahn, 2001; Wong and Sul, 2010). Oppositely it suppresses adipose lipolysis and hepatocyte gluconeogenesis. Altogether the actions of insulin favor energy storage. Once insulin reaches the cell, it binds the insulin receptor, a tyrosine kinase receptor, which starts a phosphorylation chain (Haeusler et al., 2018; Krüger et al., 2008; Menting et al., 2013). The receptor auto-phosphorylates, activating a family of adaptor proteins including the insulin receptor substrate (IRS) family (Boucher et al., 2014). These further activate downstream signaling, one of the constituents of which is the protein kinase B (AKT) family (Taniguchi et al., 2006). This family in turn affects a multitude of downstream signaling pathways, both functionally and transcriptionally. For example, it leads to increased insulin-stimulated glucose uptake through the trafficking and translocation of glucose transporter type 4 (GLUT4) vesicles to the cell surface, causing an influx of glucose into the cell and lowering blood glucose levels (Klip et al., 2014). Similarly, it stimulates amino acid uptake and protein synthesis while inhibiting protein degradation, both processes mostly regulated by AKT-activation of glycogen synthase kinase 3 (GSK3) (James et al., 2017). The transcription factor sterol regulatory element-binding protein-1c (SREBP-1c) mediates the lipid response, causing fatty acid uptake and lipogenesis, while simultaneously inhibiting lipolysis (Wong and Sul, 2010). Insulin also affects adipocyte differentiation through AKT-mediated forkhead box O1 (FoxO1) nuclear exclusion, releasing FoxO1 inhibition of adipogenic transcription factors (Nakae et al., 2003; Rosen and MacDougald, 2006). However, in the liver FoxO1 regulates gluconeogenesis, shutting down expression of necessary enzymes after nuclear

exclusion (Langlet et al., 2017). This is an immaculate display of how the same factor is used for different purposes in separate insulin-sensitive tissues.

One of the main mechanisms to shut down intracellular insulin signaling is the degradation of PIP3, and the attenuation of its production through the 3' phosphatase phosphatase and tensin homolog (PTEN) and the SH-2 containing inositol 5' polyphosphatase (SHIP) family of proteins, including 5' phosphatases SHIP1 and SHIP2 (Haeusler et al., 2018; Pal et al., 2012). To terminate signaling, the receptor internalizes inside a vesicle, and can be degraded or recycled back to the cell surface, while the bound insulin is being broken down. Most of the players in this pathway, with the exception of endocytosis regulation, are summarized in the pathway in Figure 2.

Many studies have assessed insulin signaling in a range of tissues. Most of these experiments have been performed in mouse models, by tissue-specific knockout of various players in the insulin signaling pathway, by assessing natural genetic heterogeneity, or by high-throughput screening for diabetic pathology of large collections of mouse mutants (Kubota et al., 2017; Parks et al., 2015). The most significant disruption of insulin signaling is the deletion of the insulin receptor, which has been studied in various tissues (Kubota et al., 2017). As expected these models present with deleterious effects on the animal's metabolism and overall quality of life. However, one controversial set of reports found that knocking out the adipose insulin receptor had a positive effect (Blüher et al., 2003; 2002). The fat mass in these animals was reduced, and they were protected from obesity and associated glucose intolerance. Presumably based on this protection, the mice also displayed increased median and maximum lifespan. Oppositely it has also been shown that increasing insulin sensitivity is beneficial for mice (Morley et al., 2015). Sadly, the mechanism for this protective effect has not yet been elucidated, spurring further research into this topic.

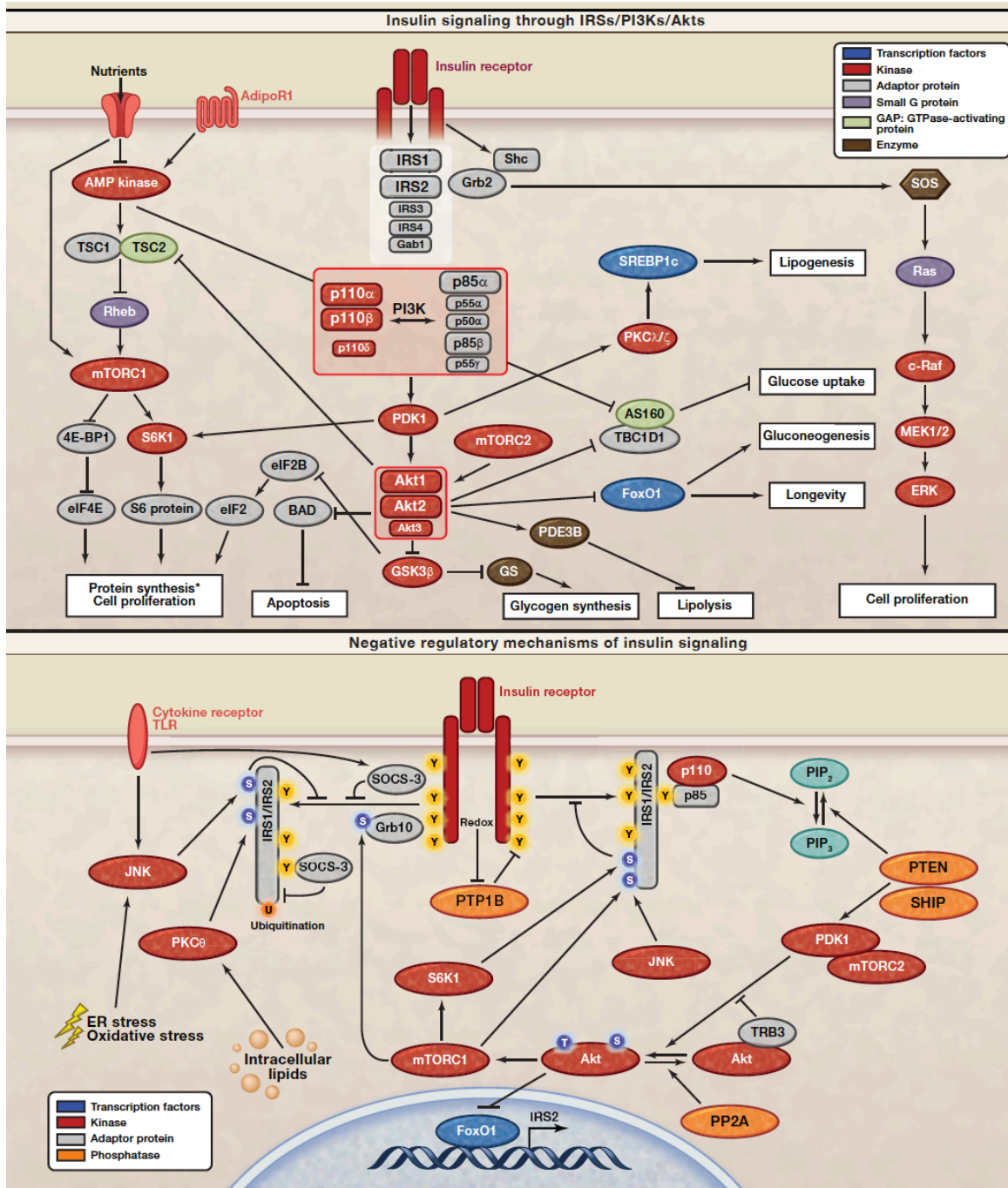


Figure 2. An overview of the insulin signaling pathway and its negative regulatory mechanisms. The top half shows the activation of main nodes (IRS family, AKT family) and the downstream metabolic effects. The bottom half shows the players acting upon the pathway to downregulate insulin signaling. (Kadowaki et al., 2012)

Inflammation

Many studies have shown that obesity is often accompanied by the development of inflammatory diseases (Tilg and Moschen, 2008). The disruption of metabolic homeostasis in nutritionally overloaded cells leads to inflammation through many paths (Chitraju et al., 2017; Hotamisligil, 2017). Due to their size, obese adipocytes experience hypoxia, undergo mechanic pressure by extracellular matrix constriction, and become insulin resistant. All of these processes activate a range of transcriptional pathways, including cytokines, chemokines and inflammatory mediators (Deng et al., 2013; Eguchi et al., 2011; Mansuy-Aubert et al., 2013; Moraes-Vieira et al., 2014; Murahovschi et al., 2014; Perry et al., 2015). The downstream effects of this transcriptional regulation consist of activation of adipose tissue-resident macrophages and infiltration of immune cells (Kälin et al., 2017; Schipper et al., 2012). Upregulated inflammatory kinases such as JNK and IKK further inhibit insulin signaling and glucose uptake, exacerbating the metabolic dysregulation (Glass and Olefsky, 2012; Osborn and Olefsky, 2012). This process also increases pro-inflammatory cytokine expression, worsening the metabolic condition of the adipocytes (Rajbhandari et al., 2018; Zhao et al., 2018). These cytokines lead to a necrosis-like state for a portion of obese adipocytes, after which macrophages will attempt to engulf the adipocyte (Lee et al., 2018). Obesity is known to be a disease associated with inflammation and necrosis, leading to a chronic illness in humans. In turn this becomes a vicious cycle with obesity exacerbating the metabolic dysfunction (Reilly and Saltiel, 2017). The only way to break this cycle is to aggressively treat either condition (Morley et al., 2015; Oral et al., 2017; Quarta et al., 2017; Wu et al., 2017).

Stem cells & genome editing

Human pluripotent stem cells (hPSCs) exist in various forms. The two major groups used in human research are embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). ESCs are derived directly from embryos, while iPSCs can be made directly from any somatic cell through reprogramming by introducing genes to revert the cell back into a pluripotent cell (Zhu and Huangfu, 2013). The use of iPSCs has been regarded as the ethical way of conducting stem cell research for human studies, due to the ease they can be derived with from readily available adult cells. ESCs on the other hand have thrown up ethical roadblocks because they have to be derived from human embryos. iPSC technology has been discovered in 2006 (Takahashi and Yamanaka, 2006). hPSCs are pluripotent because they can differentiate into all three of the major germ layers; endoderm, mesoderm and ectoderm (Zhang et al., 2012). They also have the capability to replicate indefinitely, meaning they are an infinite source of cellular material. Since the discovery of hPSCs, significant research has been done on the generation of different tissues (Turksen and Troy, 2006). To fully leverage the power of hPSCs for metabolic disease modeling it has to be possible to make mature tissues to study human diseases.

Recent advances have fully unlocked the full power of hPSCs, by enabling genome editing directly *in vitro* (Peters et al., 2013). With the advent of TALENs, and

especially the discovery of CRISPR/Cas9, the possibilities for disease modeling are now endless (Cermak et al., 2011; Cong et al., 2013; Mali et al., 2013). It is now possible to generate hPSC lines with any mutation or deletion, causing the over-activation or knockout of genes. Through endogenous cellular DNA repair mechanisms, it is possible to introduce indels, either short insertions or deletions by leveraging the error-prone non-homologous end joining repair mechanism. On the other hand, specific mutations in the genome can be made by taking advantage of homology-directed repair by presenting exogenous DNA as a repair template. These tools open up the possibility of generating any disease-causing mutation, and with the capability to differentiate genome-edited cells into any tissue, we can study human cells *in vitro* to understand the pathological mechanisms behind the disease. This means we've entered a new era, one where we are able to develop therapeutics based on mechanistic understanding of the faulty gene driving the affliction.

Adipocytes

The main energy-storing cells in the body are adipocytes, which make up the majority of the volume of fat tissue. These cells contain triglycerides as a metabolic reserve and respond to nutritional status by storing or releasing fatty acids (Rosen and Spiegelman, 2014). Adipocytes are exquisitely insulin sensitive as one of their important functions is clearing glucose from the blood upon insulin stimulation to maintain whole-body metabolic homeostasis (James et al., 1988). Subsequently they form triglycerides out of the glucose through lipogenesis and store these fatty acids in large lipid droplets (Collins et al., 2011). Not only does insulin stimulate the uptake of glucose, it also stimulates the fatty acid synthesis on both a protein and transcriptional level (Saltiel and Kahn, 2001).

Obesity is due to expansion of adipocytes, thereby increasing fat and body mass (González-Muniesa et al., 2017). The two mechanisms by which this takes place are hyperplasia and more importantly hypertrophy, which are increased proliferation and adipocyte differentiation, or cell size increase of adipocytes respectively (Rosen and MacDougald, 2006). Obesity and insulin resistance are closely related, as one will cause the other (Garg, 2006). Oversized adipocytes in obese people cause a dysregulation of hormones and lipid handling, eventually leading to adipocyte insulin resistance. On the other hand, insulin resistance causes adipocytes to continually uptake glucose and convert it into stored fat, thereby ballooning in size and causing obesity. Eventually both will lead to chronic inflammation as well (Reilly and Saltiel, 2017).

Adipocytes also have major endocrine functions, which have only recently been appreciated. Hormones released by fat are called adipokines, with most released factors related to metabolic homeostasis (Wozniak et al., 2009). The most well-known of these is leptin, which was also the first adipokine to be discovered (Zhang et al., 1995). It is critical in regulation of appetite and acts as a satiety factor, mainly by signaling through the hypothalamus in the brain (Zhang and Chua, 2017). It also regulates other metabolic processes, including the glucose-fatty acid homeostasis (Perry et al., 2018). Similarly important is adiponectin, an adipokine that also heavily modulates metabolic processes (Christou and Kiortsis, 2013; Nakashima et al.,

2008). It decreases gluconeogenesis while increasing glucose uptake, plus it promotes lipid catabolism by stimulating beta-oxidation and triglyceride clearance (Yamauchi et al., 2001). Through affecting insulin sensitivity and upregulation of uncoupling proteins, it can also modulate body weight. It confers these functions by circulating through the bloodstream and primarily acting upon skeletal muscle, the adipose tissue itself, and the liver.

Adipocytes have traditionally been arduous to study, as the excessive lipid storage complicates most biochemical assays. Additionally, it is hard to access human adipose tissue, unless the subject is obese. Therefore, most human research has only focused on unhealthy adipose tissue. Due to these restrictions, most adipocyte biology has been studied in mice, considering the tractability of the system and the ability to study multi-organ interactions. However, especially in metabolic research findings are often not consistent between mice and men, owing to differing metabolic biology. It has always been a focus of the field to study human adipocytes in both healthy and metabolically dysregulated states. With the advent of iPSC technology, it has become possible to study human adipocytes in a dish. The creation of a differentiation protocol from hPSCs to adipocytes has allowed us unlimited access to biological material for human fat (Ahfeldt et al., 2012). These adipocytes have proven to be transcriptionally extraordinary similar to human adipocytes, display appropriate mitochondrial metabolism, and can be used to study glucose and lipid metabolism (Ahfeldt et al., 2012; Ding et al., 2013). With genome editing we can also model metabolic genetic diseases found in patients, and investigate the mechanisms underlying these pathological states. These adipocytes can also be implanted into mice to assess their function in an organismal setting and interrogate various genetics and functionalities of the adipocytes.

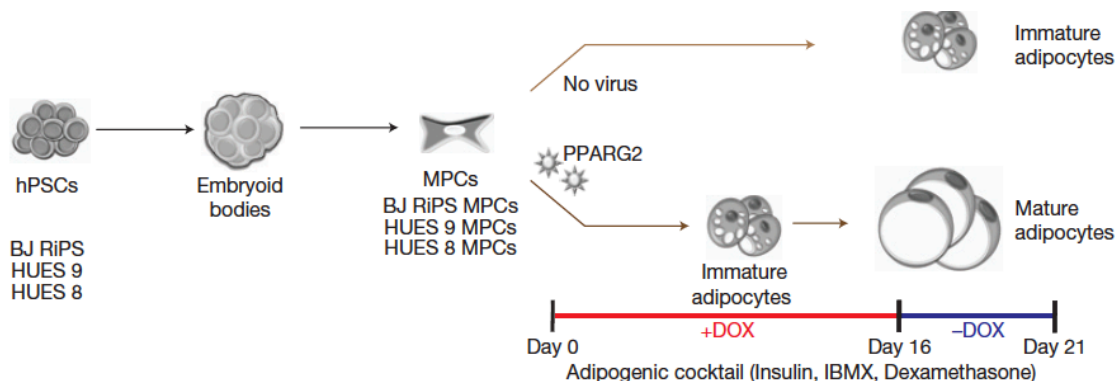


Figure 3. Experimental scheme for the differentiation of hPSCs into adipocytes. hPSCs are differentiated into embryoid bodies, re-plated and passaged to generate mesenchymal progenitor cells. These cells are then transduced with *PPARG2* lentivirus. Adipocytes are differentiated over a 3 week period in adipogenic medium before analysis. (Ahfeldt et al., 2012)

Hepatocytes

Hepatocytes, comprising the majority of the liver's mass, are the cell type responsible for metabolic functions of the organ (Rui, 2014). The hepatocyte is the main source of serum albumin, fibrinogen, and clotting factors. This excessive protein production strains the cells, leading to polyploidy and multinucleate hepatocytes being common (Gentric and Desdouets, 2014). Hepatocytes also regulate lipid metabolism, for example by synthesis and recycling of lipoproteins (Nguyen et al., 2008). They metabolize chylomicrons, synthesize cholesterol, release low-density lipoprotein and take up high-density lipoprotein particles. The liver is also the sole producer of bile salts (Chiang, 2017). Lastly, hepatocytes play an instrumental role in detoxifying the body by metabolizing and inactivating compounds such as drugs, but also endogenous steroids (Guillouzo et al., 1993; Waxman et al., 1988).

One contributor to lipid metabolism in hepatocytes specifically is the 1p13 rs12740374 common noncoding polymorphism. In a GWAS this genetic variant was found to be associated with both plasma low-density lipoprotein cholesterol (LDL-C) and myocardial infarction risk (Teslovich et al., 2010). Through creation of a CCAAT/enhancer binding protein transcription factor binding site it alters the expression of the *SORT1* gene. It has been shown that Sort1 alters plasma LDL-C and very low-density lipoprotein (VLDL) particle levels by regulating hepatic VLDL secretion (Musunuru et al., 2010).

Compared to adipocytes, more research has been done on differentiating hepatocytes from hPSCs. Many protocols have been developed which generate liver cells (Ding et al., 2013; Hannan et al., 2013; Loh et al., 2014). Most do not fully recapitulate the *in vivo* hepatocytes found in the human body, but the resultant cells are sufficiently functional to model glucose and lipid metabolism (Ding et al., 2013). Various approaches have been taken to generate a more mature hepatocyte population, including small molecule additions to the differentiation protocol, specialized culture conditions or hepatocyte purification steps (Mallanna et al., 2016; March et al., 2015; Schepers et al., 2016; Shan et al., 2013).

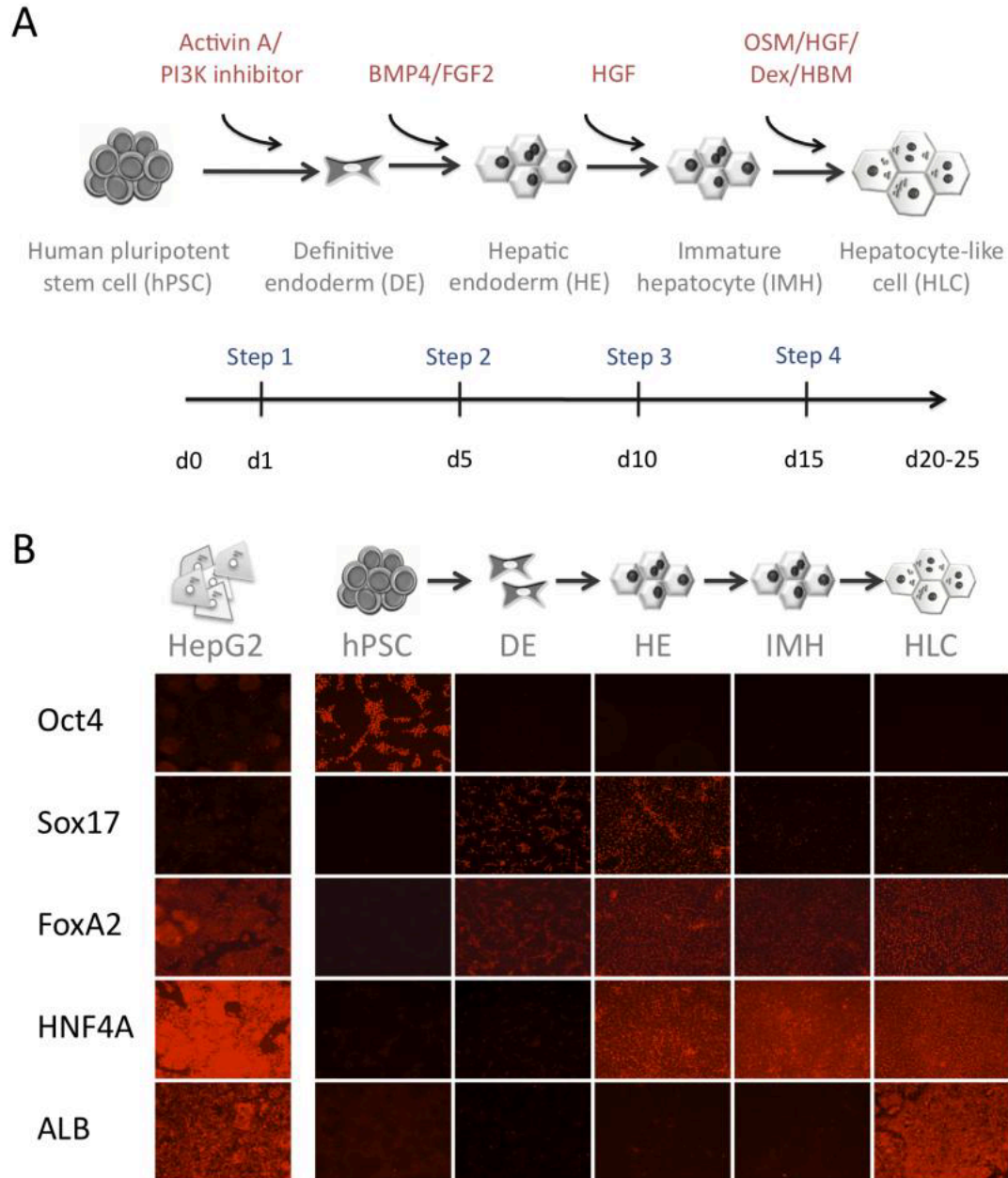


Figure 4. Differentiation scheme and experimental validation of hepatocytes. A) Differentiation protocol of hPSCs into hepatocytes in a four-stage progression. B) Representative hepatocyte differentiation with marker staining at various stages of progress. Cultured human HepG2 cells are used as a comparison. (Ding et al., 2013)

Aim and outline of the thesis

In this thesis we aim to explore the mechanisms of metabolic disease. Given the incredibly broad range of pathologies in this class, there are two main areas we focus on. The first is adipocyte metabolism, which we explore in chapter 2 through 4. In chapter 2 we investigate lipodystrophy by generating iPSCs from patients with a *LMNA* mutation. These cells have a deficiency in adipocyte differentiation, lipolysis, mitochondrial metabolism and dysregulated insulin signaling that promotes adipose wasting. This in turn copies the whole-body phenotype of the patients. In chapter 3 we delve into the mechanism of insulin signaling, by examining the implications of knocking out the insulin receptor specifically in adipocytes. In a murine model lacking the adipose *INSR* we observe severe effects on body weight, driven by a lack of adipocytes, which leads to glucose metabolism dysfunction. While these animals are protected from high fat diet-induced obesity, the severity of metabolic complications is such that it causes a dramatically reduced lifespan. Chapter 4 digs into the relationship between obesity and inflammation, as we attempt to identify a transcription factor that is upregulated during obesity. We find *IRF1* to be highly expressed in obese adipocytes, and *in vitro* differentiated adipocytes overexpressing this factor recapitulate obesity phenotypes. The *IRF1* adipocytes have more unilocular lipid droplets and have attenuated insulin signaling. When implanted into mice they also attract more inflammatory immune cells. This establishes a gene expression link between inflammation and obesity. The second half of this thesis switches focus to explore liver metabolism. We have optimized a differentiation protocol for hepatocytes in chapter 5. Not only have we standardized the protocol, we also make the case for purification based on surface marker ASGPR1 cell sorting. These purified hepatocytes mimic primary human hepatocytes much closer compared to unpurified bulk hepatocytes. This new protocol sets up the tools required for chapter 6. For this project we needed a supremely robust differentiation protocol to ensure the purity of 68 hepatocyte differentiations of 34 separate iPSC lines. The concept of this study was to recapitulate *in vitro* the findings of a genome-wide association study in which a single nucleotide polymorphism affected *SORT1* expression specifically in hepatocytes. We differentiated hepatocytes with our established protocol, and pairwise differentiated adipocytes as a negative control. The iPSC lines used for this study were generated from 17 donors with a major genotype for the genetic variation affecting *SORT1*, and 17 donors with a minor genotype. We were able to confirm the GWAS findings and demonstrate that it is in fact possible to model SNPs *in vitro* using iPSCs. As a follow-up experiment we identified hepatic metabolites that were significantly regulated by the genotype of these cells. Finally, at the end of this thesis we present a discussion of the results, the implications for metabolic disease, and the outlook on future research with an aim towards therapeutic interventions for obesity, T2D, CVD and insulin resistance.

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FPLD2 *LMNA* mutation R482W dysregulates iPSC-derived adipocyte function and lipid metabolism



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ABSTRACT

Lipodystrophies are disorders that directly affect lipid metabolism and storage. Familial partial lipodystrophy type 2 (FPLD2) is caused by an autosomal dominant mutation in the *LMNA* gene. FPLD2 is characterized by abnormal adipose tissue distribution. This leads to metabolic deficiencies, such as insulin-resistant diabetes mellitus and hypertriglyceridemia. Here we have derived iPSC lines from two individuals diagnosed with FPLD2, and differentiated these cells into adipocytes. Adipogenesis and certain adipocyte functions are impaired in FPLD2-adipocytes. Consistent with the lipodystrophic phenotype, FPLD2-adipocytes appear to accumulate markers of autophagy and catabolize triglycerides at higher levels than control adipocytes. These data are suggestive of a mechanism causing the lack of adipose tissue in FPLD2 patients.

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1. Introduction

Mutations in the *LMNA* gene result in a family of syndromes known as laminopathies, including premature aging syndromes, myopathies, neuropathies, and lipodystrophy. *LMNA* lipodystrophy is known as familial partial lipodystrophy type 2 (FPLD2), an autosomal dominant genetic disorder [1]. FPLD2 is characterized by atypical subcutaneous adipose tissue distribution; specifically atrophy of subcutaneous adipose in the trunk and limbs, and excess accumulation of supraclavicular fat [2]. This dearth of subcutaneous adipose tissue drives systemic insulin resistance and hyperinsulinemia, potentiating progression to diabetes mellitus as well as hypertriglyceridemia followed by early atherosclerosis [3]. Reduced adiposity also leads to decreased serum concentrations of the adipokine adiponectin [4].

The *LMNA* gene resides on chromosome 1q21, encoding nuclear lamins A and C [5]. The most common variant of *LMNA* associated with FPLD2 is a missense mutation, R482W. The R482W mutation is solely associated with FPLD2; carriers display no disorders associated with other laminopathies [6]. The mutated residue lies in the C-terminal globular tail of lamin A where it does not alter the

three-dimensional structure of the protein [7]. This mutation causes structural defects in the nuclear envelope as a result of disrupted protein-protein interactions [8,9].

We have reprogrammed induced pluripotent stem cell (iPSC) lines derived from individuals carrying the R482W mutation of *LMNA*. We differentiated the iPSCs into adipocytes (FPLD2-adipocytes) to investigate the cellular underpinnings of FPLD2. These *LMNA* R482W iPSC lines display reduced adipogenesis, altered insulin signaling as well as markers of increased autophagy. FPLD2-adipocytes exhibit increased basal and stimulated lipolysis as well as increased maximal mitochondrial activity. These molecular abnormalities in iPSC-derived adipocytes may be connected to the disease pathogenesis.

2. Results

2.1. Generating patient iPS lines

The aim of this study was to compare adipocytes derived from carriers of the *LMNA* R482W mutation with those of healthy controls (Fig. 1A). Towards this end we reprogrammed iPSCs from

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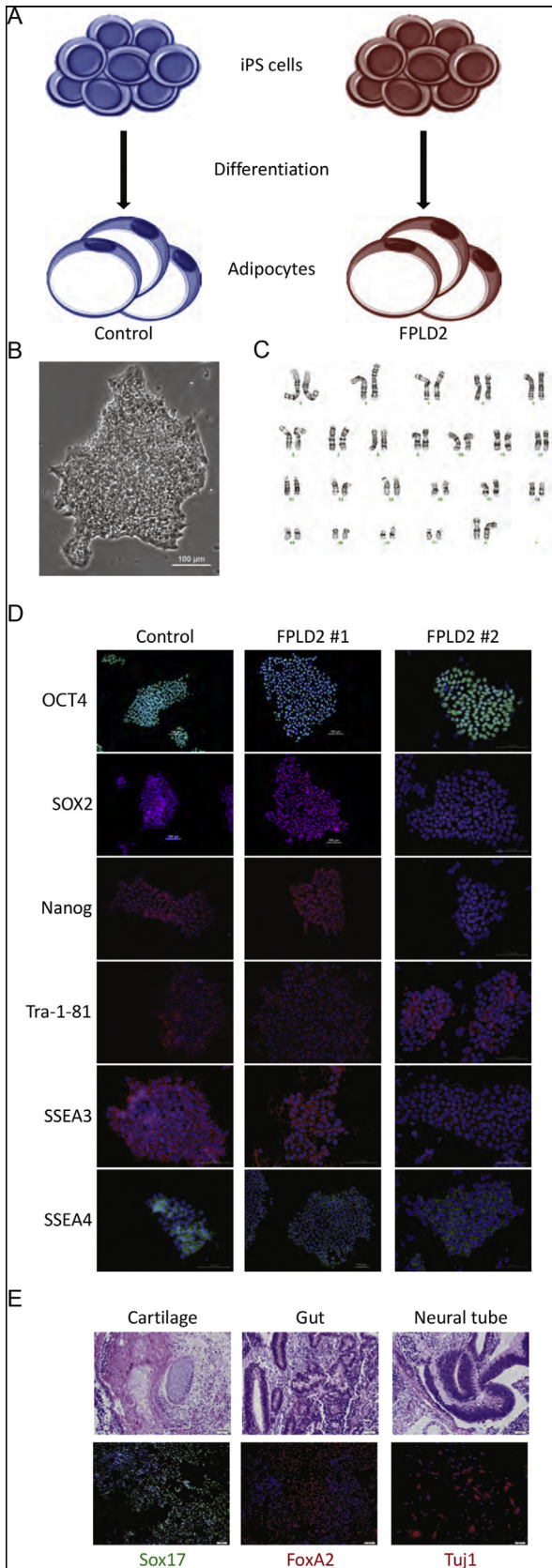


Fig. 1. iPS cell characterization. A) Experimental outline of the study. B) Representative iPS cell colony. C) Karyotype results of one of the FPLD2 lines. D) Immunostaining for pluripotency markers of the WT control line and both FPLD2 lines. E) Results of the teratoma assay exhibiting all three germ layers by H&E staining and immunofluorescence.

dermal fibroblasts obtained from two FPLD2 individuals harboring the R482W missense mutation in the *LMNA* gene. The cells displayed normal pluripotent stem cell morphology (Fig. 1B) and carried no chromosomal abnormalities (Fig. 1C). The cells also expressed pluripotency markers (OCT4, Nanog, Tra-1-81, SSEA3, and SSEA4) as evidenced by immunostaining (Fig. 1D). R482W FPLD2 iPS cells generated cells from all three germ layers in an *in vivo* teratoma formation assay, a hallmark of pluripotent cells (Fig. 1E).

2.2. FPLD2 lines have diminished capability for differentiation

Our previously established adipocyte differentiation protocol was employed to produce iPS cell-derived white adipocytes (iPSC-WAT) [10]. Differentiation efficiency was evaluated by quantification of nuclei positive for the transcription factor and adipogenic master regulator CCAAT/Enhancer Binding Protein Alpha (C/EBP-alpha) [11]. Lipid droplets were imaged using a fluorescent neutral lipid dye (Fig. 2A). Both FPLD2-adipocytes and control cells were capable of adipogenesis, however the differentiation efficiency was decreased in the FPLD2 cell lines (Fig. 2B). Differentiated adipocytes also expressed the lipid droplet coating protein perilipin-1, while lamin A protein is detected in the nuclei of both FPLD2 and wild-type adipocytes (Fig. 2C). When partially differentiated cells were injected into the immunodeficient Rag2^{-/-};IL2^γC mouse model, both groups of cells generated adipocytes. Adipocyte transplants were excised and imaged six weeks after implantation (Fig. 2D). Implants composed of control cells develop mature adipocytes with monolocular lipid droplets while the FPLD2 cells exhibited a disorganized structure and smaller lipid droplets.

2.3. FPLD2-adipocytes express decreased adipocyte markers and are resistant to insulin treatment

Correlating with decreased adipocyte differentiation, steady-state mRNA levels of mature adipocyte markers were also slightly decreased in FPLD2-adipocytes, including *AdipoQ*, *FABP4*, *HLS*, and *GLUT4* (Fig. 3A). To corroborate these findings we measured the cellular accumulation (Fig. 3B) and secretion (Fig. 3C) of adiponectin in FPLD2-adipocytes by immunoblotting and enzyme-linked immunosorbent assay (ELISA). Both of these measures indicated a significant reduction in adiponectin production, in agreement with decreased *ADIPOQ* gene expression. These findings are consistent with the clinical observation of reduced adiponectin in the serum of FPLD2 patients [4].

Next, we tested the response of these adipocytes to insulin stimulation. Phosphorylation of AKT and ERK1/2 in response to insulin appeared decreased in FPLD2-adipocytes (Fig. 3D and E). The reduced signal transduction downstream of the insulin receptor observed in *LMNA* R482W mutant cells requires additional investigation to determine the exact molecular basis, but is consistent with previous reports of insulin resistance in FPLD2 patients [3].

2.4. Functional deficiencies of FPLD2 adipocytes

Since the mechanism of fat loss in FPLD2 is not well understood, we performed lipolysis assays using iPS cell-derived adipocytes. FPLD2-adipocytes displayed increased lipolysis both under basal conditions and during isoproterenol stimulation (Fig. 4A). Elevated lipolysis lead to increased fatty acid utilization by FPLD2-adipocyte mitochondria; and the observed maximal oxygen consumption rate was heightened in FPLD2-adipocytes (Fig. 4B).

Furthermore we investigated autophagic flux in iPS-WAT. The observed ratio of LC3-II to LC3-I increased in the FPLD2 lines as measured by western blot, suggesting an increase in

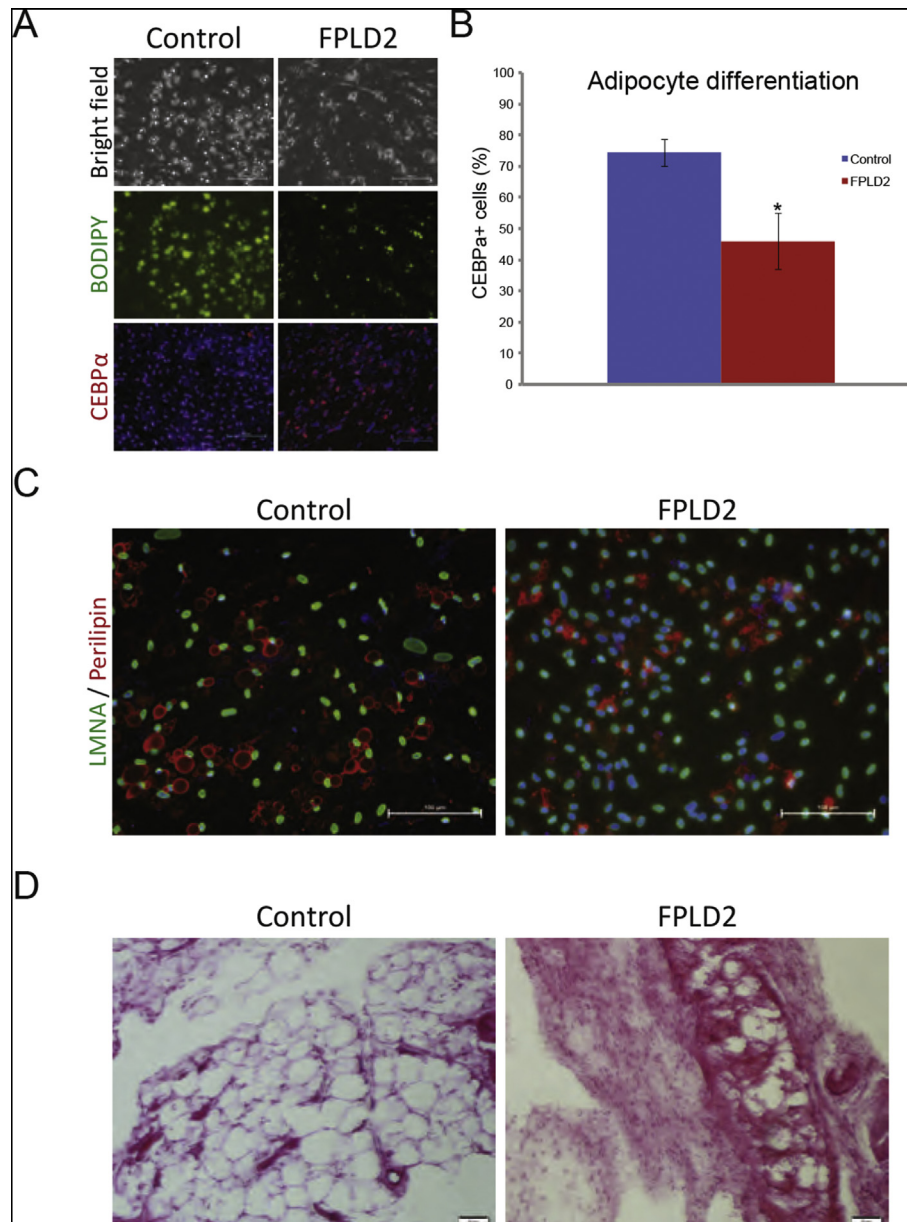


Fig. 2. Adipocyte differentiation and quality control. A) Brightfield imaging, BODIPY and CEBP α immunofluorescence of healthy and FPLD2 lines. DAPI is used as a nuclear co-stain. B) Quantification of the CEBP α -positive nuclei. C) Immunostaining of PLIN1 and LMNA. DAPI is used as a nuclear co-stain. D) Images of the adipose depots formed after injecting healthy or FPLD2 adipocytes into Rag2 $^{-/-};\gamma$ C mice. Error bars, standard deviation. * = p -value ≤ 0.05 .

autophagosome formation (Fig. 4C and D). Concomitantly, the steady-state protein level of the autophagy marker ATG7 was increased in FPLD2 lines as measured by ELISA (Fig. 4E).

3. Discussion

The LMNA R482W mutation appears to manifest a number of cellular and molecular defects in iPSC-derived adipocytes. The clinical hallmarks of FPLD2 are lipoatrophy in the subcutaneous adipose depots of the limbs and trunk, insulin resistance, and decreased serum adiponectin levels [3,4]. FPLD2-iPSC lines are capable of adipocyte differentiation, albeit at a lower efficiency than control iPSC lines. Reduced differentiation efficiency is coupled with reduced mRNA expression of adipocyte markers. In

accord with the clinical phenotype, adiponectin accumulation and secretion are significantly reduced in FPLD2-adipocytes. Additionally, insulin-dependent phosphorylation of AKT2 and ERK1/2 was impaired in FPLD2-adipocytes, which could explain the insulin resistance in FPLD2 adipocytes. These cellular phenotypes recapitulate several key clinical phenotypes observed in FPLD2 patients.

The reduction of adipose tissue in FPLD2 *in vivo* could be driven by a combination of increased lipolysis and decreased lipogenesis, as observed in this study. Elevated lipolysis in FPLD2-adipocytes liberates free fatty acids from intracellular stores, providing the substrate for increased oxygen consumption. We speculate that the coupling of increased fatty acid catabolism with decreased *de novo* lipogenesis, normally driven by insulin signaling, may shift the lipid metabolism balance in FPLD2-adipocytes [12]. This shift in lipid

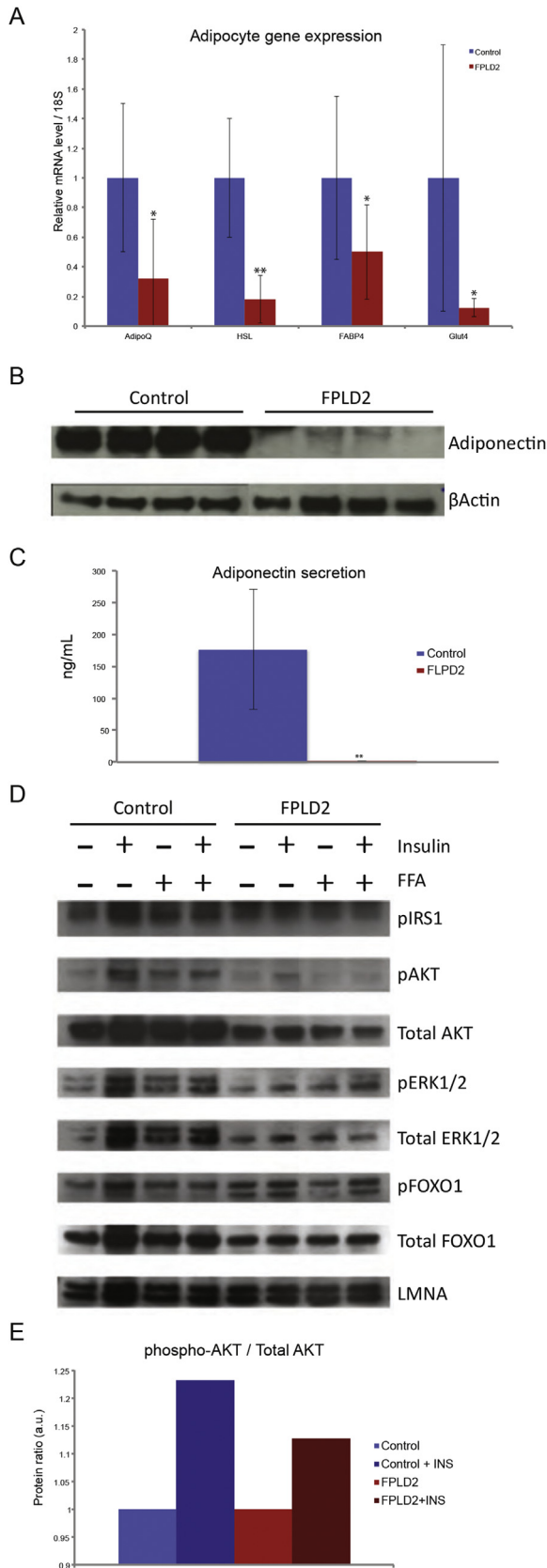


Fig. 3. FPLD2 adipocytes lack key adipocyte features and are insulin resistant. A) qPCR for *ADIPOQ*, *HSL*, *FABP4* and *GLUT4*, normalized to healthy lines and *18S* used as an internal control. B) Western blot for intracellular adiponectin protein. Beta-actin is used as a loading control. C) Adiponectin secretion from the adipocytes is measured by ELISA. D) Western blot for insulin signaling pathway proteins. Antibodies were used

metabolism could contribute to the observed localized lipodystrophy in FPLD2. Our data suggests that this metabolic shift towards fatty acid utilization may be coordinated by autophagy. Increased markers of autophagy in FPLD2-adipocytes are most parsimoniously explained via an upregulation of lipophagy or chaperone-mediated autophagy of perilipin, which would result in increased lipolysis [13–15].

4. Methods

4.1. iPS derivation

Human fibroblasts with LMNA mutations were provided by Dr. Vigouroux [18] and reprogrammed using retroviral vectors [19]. BJ RiPS were used as control iPS cell line [20].

4.2. Immunofluorescence

The following antibodies and dilutions were used: α -CEBPA 1:100 (Santa Cruz #sc-61), α -PLIN1 1:200 (Novus Biologicals), FABP4 (R&D Systems #PPS068), Oct4 (Santa Cruz #sc-8628), Nanog (Millipore #AB9220), Sox2 (Abcam #ab15830), SSEA3 (Millipore #MAB4303), SSEA4 (Millipore #MAB4304), Tra-1-81 (Millipore #MAB4381). Lipid droplets were stained using BoDIPY neutral lipid dye, cell nuclei with DAPI. Alexa Fluor secondary antibodies were used (Invitrogen). Images were acquired using a Nikon Eclipse Ti-S microscope. Both NIS-Elements and Image J software packages were used for image analysis.

After excision teratomas were formaldehyde-fixed, embedded in paraffin, sectioned and stained with hematoxylin and eosin, or with the following antibodies: FoxA2 (Abcam #ab40874), Sox17 (R&D Systems #AF1924), Tuj1 (Covance #MMS-435P).

4.3. Teratoma formation assay

The teratoma assay was performed as in Ref. [21].

4.4. In vivo adipocyte transplantation

Control or FPLD2 cells were differentiated for 14 days. Differentiated cells were injected subcutaneously into Rag2^{-/-};IL2 γ C mice from The Jackson Laboratory. Four weeks after transplantation, mice were killed to collect fat implants. Tissues at the transplantation site were formaldehyde-fixed, embedded in paraffin and hematoxylin and eosin stained after sectioning [22].

4.5. Adipocyte differentiation

Differentiation was performed as in Ref. [10]. Briefly, iPS cells were differentiated into mesenchymal progenitor cells (MPCs) through embryoid bodies. Lentiviral delivery of a doxycycline-inducible *PPAR* γ 2 programmed the MPCs into mature white adipocytes.

4.6. Quantitative polymerase chain reaction (qPCR)

Total RNA from iPSC-adipocytes was extracted with Trizol (Invitrogen) and purified using the RNeasy mini kit (Qiagen) according to the manufacturer's instructions. The RNA yield was

against phosphorylated IRS1, phosphorylated AKT, total AKT, phosphorylated ERK1/2, total ERK1/2, phosphorylated FoxO1, and total FoxO1. LMNA was used as a loading control. E) Ratio of phosphorylated over total AKT, data taken from panel D. Samples have been normalized by the loading control and differentiation efficiency. Error bars, standard deviation. * = p-value $\leq$ 0.05, ** = p-value $\leq$ 0.01.

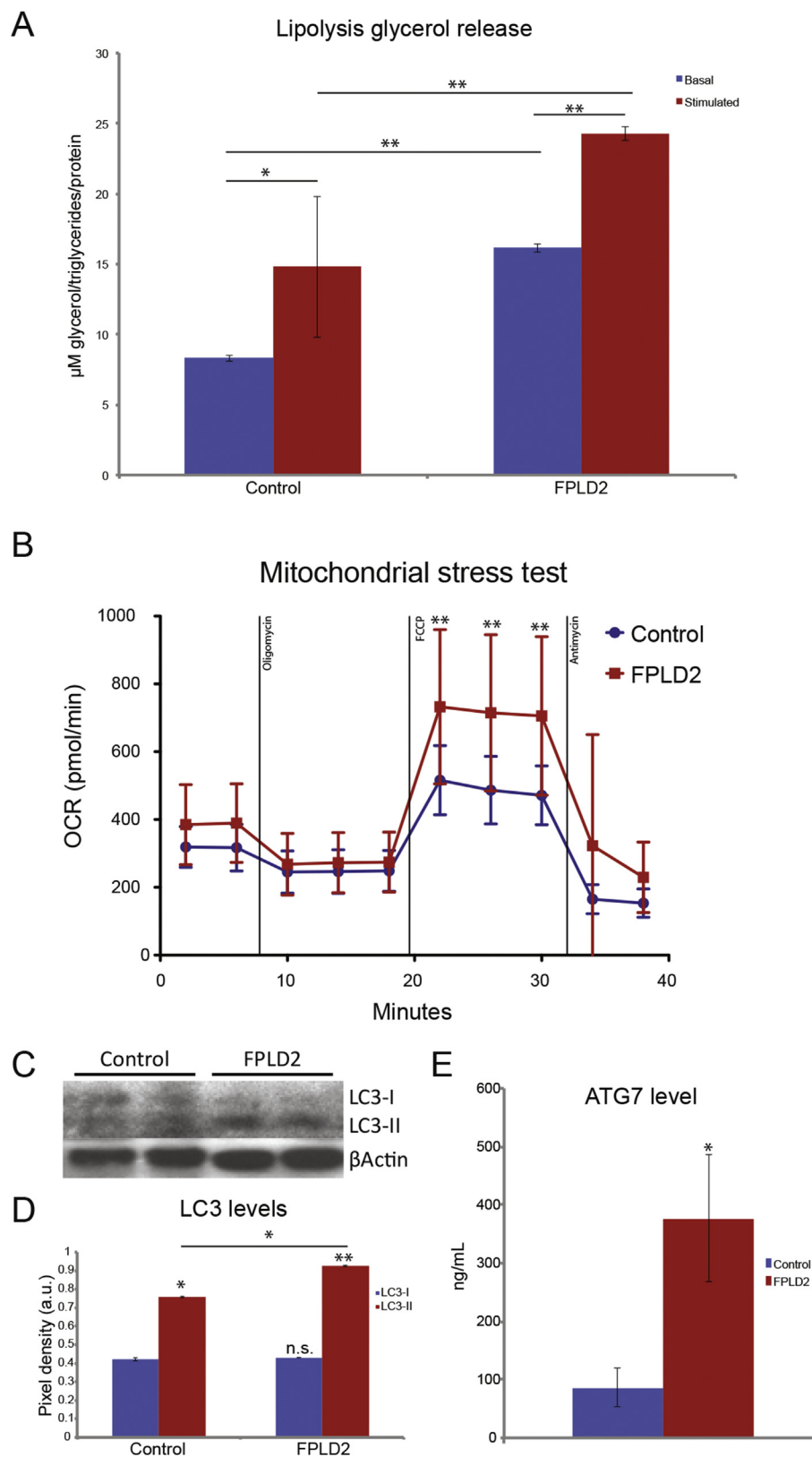


Fig. 4. FPLD2 adipocytes increase lipolysis as well as oxygen consumption and accumulate markers of autophagy. A) Lipolysis assay showing basal and isoproterenol-stimulated release of glycerol. B) Oxygen consumption during the mitochondrial stress test on a Seahorse XF analyzer. C) Western blot of LC3-I and LC3-II. Beta-actin is used as a loading control. D) Quantification of LC3 levels shown in panel C. E) ATG7 levels determined by ELISA. Error bars, standard deviation. * = p-value ≤ 0.05 , ** = p-value ≤ 0.01 .

determined using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). Total RNA (1 µg) was converted to cDNA using the Superscript First-Strand kit (Invitrogen). RT-qPCR was carried out using a Realplex Mastercycler (Eppendorf) with the Quantifast-SYBR Green PCR mix (Qiagen) with 1 µl cDNA per reaction. qPCR primer sequences used;

ADIPOQ:	F-gatgaagtcctgtcttgaagg	R-cagcacttagatggaggttg.
GLUT4:	F-ccaacagataggctccgaaga	R-accgcagagaacacagcaa.
HSL:	F-gcctcagttccgaaaacca	R-caccagcggaagtctc.
FABP4:	F-tcatgaaaggcgctacttc	R-gcttgctaatacagggaaca.
18S:	F-accgcagctaggaataatgga	R-gcctcagttccgaaaacca.

4.7. Western blotting

Adipocytes were starved overnight in DMEM containing 2% fatty acid free BSA. To induce insulin resistance, FFA mixture (lauric, myristic, linoleic, oleic and arachidonic acids from Sigma) was used at 1 mM for 1 h. Insulin stimulation was carried out at 100 nM for 20 min. Cells were lysed in cold RIPA buffer (60 mM Tris-HCl at pH 7.4, 150 mM NaCl, 0.25% SDS and 1% NP40) containing 10 mM NaF, 1 mM Na3VO4 and complete protease inhibitor (Roche). Western blot analysis was carried out using the following antibodies: total AKT (Cell Signaling #9272), phospho-Ser-473 AKT (Cell Signaling #9271), phospho-IRS1 (Millipore #07-247), IRS1 (Santa Cruz #sc-559), Adiponectin (Abcam #ab22554), Beta-actin (Sigma #A5316), and lamin A/C (Fisher/Millipore #3211). Densitometry was performed using the gel analysis function of the ImageJ software package [23].

4.8. ELISA

Culture media was collected and ELISA was performed on a Luminex 200 analyzer using the Adiponectin or ATG7 ELISA kit (Millipore) following the manufacturer's instructions. ELISA readings from culture media alone were subtracted from each experimental sample as a background control, and protein concentration was used to normalize.

4.9. Lipolysis

To measure lipolysis activity, day 21 adipocytes differentiated from both healthy and FPLD2 iPSCs were starved in DMEM containing 1% FBS for 1 h. Cells were then incubated in HBSS (Hank's balanced salt solution) with 2% fatty-acid-free BSA alone, with 1–10 µM isoproterenol. The culture medium was collected for glycerol measurement using the free glycerol reagent (Sigma #F6428). Protein concentrations used to normalize glycerol content were measured using the Bradford protein assay (BioRad). Glycerol release was expressed in micrograms of glycerol per milligram of total protein.

4.10. Measurement of cellular oxygen consumption rate

Cells were plated in 0.1% gelatin-coated XF 24-well cell culture microplates (Seahorse Bioscience) and differentiated into adipocytes. Cells were incubated in pre-warmed unbuffered DMEM medium (DMEM containing 2 mM GlutaMAX, 1 mM sodium pyruvate, 1.85 g/L NaCl and 25 mM glucose) for 1 h. The oxygen consumption was measured by the XF24 extracellular flux analyzer (Seahorse Biosciences). Oxidative capacity was profiled by injecting perturbation drugs, 2 µM oligomycin, 0.5 µM FCCP (carbonyl cyanide-p-trifluoromethoxyphenylhydrazone) and 5 µM antimycin

A, in succession. OCR was determined by plotting the oxygen tension of the medium in the chamber as a function of time.

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Transparency document

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Adipocyte insulin receptor activity maintains adipose tissue mass and lifespan



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ABSTRACT

Type 2 diabetes follows a well-defined progressive pathogenesis, beginning with insulin resistance in metabolic tissues such as the adipose. Intracellular signaling downstream of insulin receptor activation regulates critical metabolic functions of adipose tissue, including glucose uptake, lipogenesis, lipolysis and adipokine secretion.

Previous studies have used the *aP2* promoter to drive Cre recombinase expression in adipose tissue. Insulin receptor (IR) knockout mice created using this *aP2*-Cre strategy (FIRKO mice) were protected from obesity and glucose intolerance. Later studies demonstrated the promiscuity of the *aP2* promoter, casting doubts upon the tissue specificity of *aP2*-Cre models. It is our goal to use the increased precision of the *Adipoq* promoter to investigate adipocyte-specific IR function. Towards this end we generated an adipocyte-specific IR knockout (AIRKO) mouse using an *Adipoq*-driven Cre recombinase.

Here we report AIRKO mice are less insulin sensitive throughout life, and less glucose tolerant than wild-type (WT) littermates at the age of 16 weeks. In contrast to WT littermates, the insulin sensitivity of AIRKO mice is unaffected by age or dietary regimen. At any age, AIRKO mice are comparably insulin resistant to old or obese WT mice and have a significantly reduced lifespan. Similar results were obtained when these phenotypes were re-examined in FIRKO mice. We also found that the AIRKO mouse is protected from high-fat diet-induced weight gain, corresponding with a 90% reduction in tissue weight of major adipose depots compared to WT littermates. Adipose tissue mass reduction is accompanied by hepatomegaly and increased hepatic steatosis.

These data indicate that adipocyte IR function is crucial to systemic energy metabolism and has profound effects on adiposity, hepatic homeostasis and lifespan.

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1. Introduction

Insulin resistance has reached epidemic levels, contributing to an increase in the prevalence of obesity, type 2 diabetes (T2D), and cardiovascular disease [1]. The onset of insulin resistance in metabolic tissues precipitates a cascade of pathologies, culminating in failure of glucose homeostasis and exhaustion of insulin-producing pancreatic beta cells. Insulin signaling through the

insulin receptor (IR) plays a pivotal role in the differentiation, development and cellular function of both white and brown adipose tissue. Not only does the IR regulate lipid metabolism through suppression of lipolysis and induction of lipogenesis in adipocytes, it also stimulates glucose uptake [2]. Adipose tissue regulates systemic energy metabolism by secreting hormones, called adipokines. This endocrine function of adipose tissue is in part regulated by insulin through the IR signaling pathway [3]. Adipocyte insulin resistance can affect each of these functional capacities, with adverse consequences for systemic metabolic health [4].

Previous studies have examined the role of insulin signaling in the adipose tissue, utilizing a “fat-specific” insulin receptor knockout (FIRKO) mouse, generated using an *aP2* promoter-driven Cre recombinase [5]. Fat mass was reduced in FIRKO mice, and

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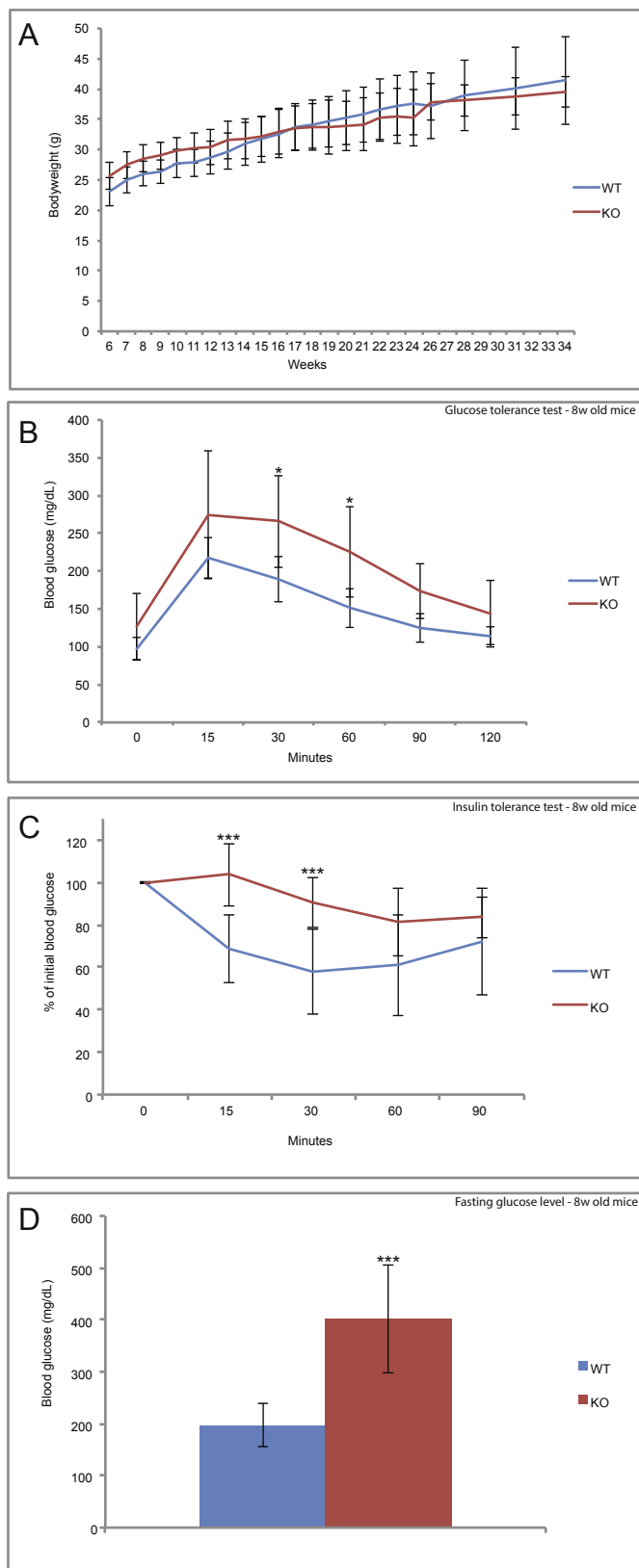


Fig. 1. Characterizing the AIRKO mouse. A) Bodyweight measurements over time from AIRKO and WT littermates. B) GTT performed on 2-month-old mice. C) ITT performed on 2-month-old mice. D) Fasting blood glucose of 4-h fasted 2-month-old mice. Mouse groups N = 13 WT mice and 11 KO mice. Error bars, standard deviation. * = p-value $\leq$ 0.05, ** = p-value $\leq$ 0.01, *** = p-value $\leq$ 0.001.

these mice were protected from obesity and associated glucose intolerance. FIRKO mice have an increased median and maximum life span, presumably as a product of protection from obesity and glucose intolerance [6].

One drawback of these studies was that the authors employed an *aP2* promoter-driven Cre. Since publication of the FIRKO mouse studies, other groups have demonstrated the promiscuity of the *aP2* promoter, driving expression of transgenes in several off-target tissues [7–10]. As the *aP2* promoter driven Cre is not adipose tissue specific, this can lead to confounding results, as phenotypes may be the product of knockout in other tissues.

The reported phenotypes of the FIRKO mouse were surprising, given the importance of the IR in adipose tissue function. We re-examined the phenotypes of FIRKO mice and to investigate this in further depth, we use the *Adipoq* promoter-driven Cre transgene. *Adipoq* expression is restricted to mature fat, mitigating effects of IR knockout on adipogenesis [11]. The resulting Adipocyte-specific Insulin Receptor Knockout (AIRKO) and FIRKO mice are protected from the exacerbation of glucose intolerance by age and obesity. They do suffer from intrinsic metabolic defects. Adipose tissue mass is significantly reduced, and compensation for the lack of adipose tissue results in hepatomegaly and increased triglyceride accumulation in the liver. AIRKO and FIRKO mouse lifespan is significantly reduced, indicating that adipose IR activity is critical for extending lifespan by promoting systemic metabolic health.

2. Materials and methods

2.1. Animals and diets

The Harvard University IACUC approved all protocols. IRLox mice were bred with transgenic mice that express Cre recombinase driven by the adipose-specific *Adipoq* promoter/enhancer region (AIRKO mice), or the *aP2* enhancer (FIRKO mice). AIRKO and FIRKO mice were obtained with the expected Mendelian frequency and displayed normal development. All mouse strains were acquired from The Jackson Laboratory. Mice were housed on a 12 h light/dark cycle with ad libitum access to water and food. For high-fat diet (HFD) experiments the mice were fed chow diet until 10 weeks of age, and at that point transferred to a 60% high fat diet (Harlan Teklad) for an additional 14 weeks.

2.2. Glucose and insulin tolerance tests

Glucose tolerance tests (GTT) were performed on overnight-fasted mice injected intraperitoneally (IP) with 1 g of D-glucose per kg of bodyweight. Insulin tolerance tests (ITT) were performed on 4-h fasted mice injected IP with 1 IU/kg bodyweight of insulin (Sigma). Pyruvate tolerance tests (PTT) were performed on overnight-fasted mice injected IP with 1 g of pyruvate per kg of bodyweight. During GTT, ITT, and PTT, blood glucose levels were measured using the OneTouch Ultra 2 system (OneTouch) at 0, 15, 30, 60, and 90 min post-injection. During GTT and PTT, blood glucose was also measured at 120 min.

2.3. Tissue analysis

Tissues were dissected, photographed, weighed and fixed in 4% paraformaldehyde (Electron Microscopy Sciences). Tissues were paraffinized and cut into sections by the Harvard Stem Cell and Regenerative Biology Histology-Immunohistochemistry core laboratory using the method described in Ref. [12]. Sections were stained with hematoxylin and eosin [13] and imaged on a BX53 microscope (Olympus).

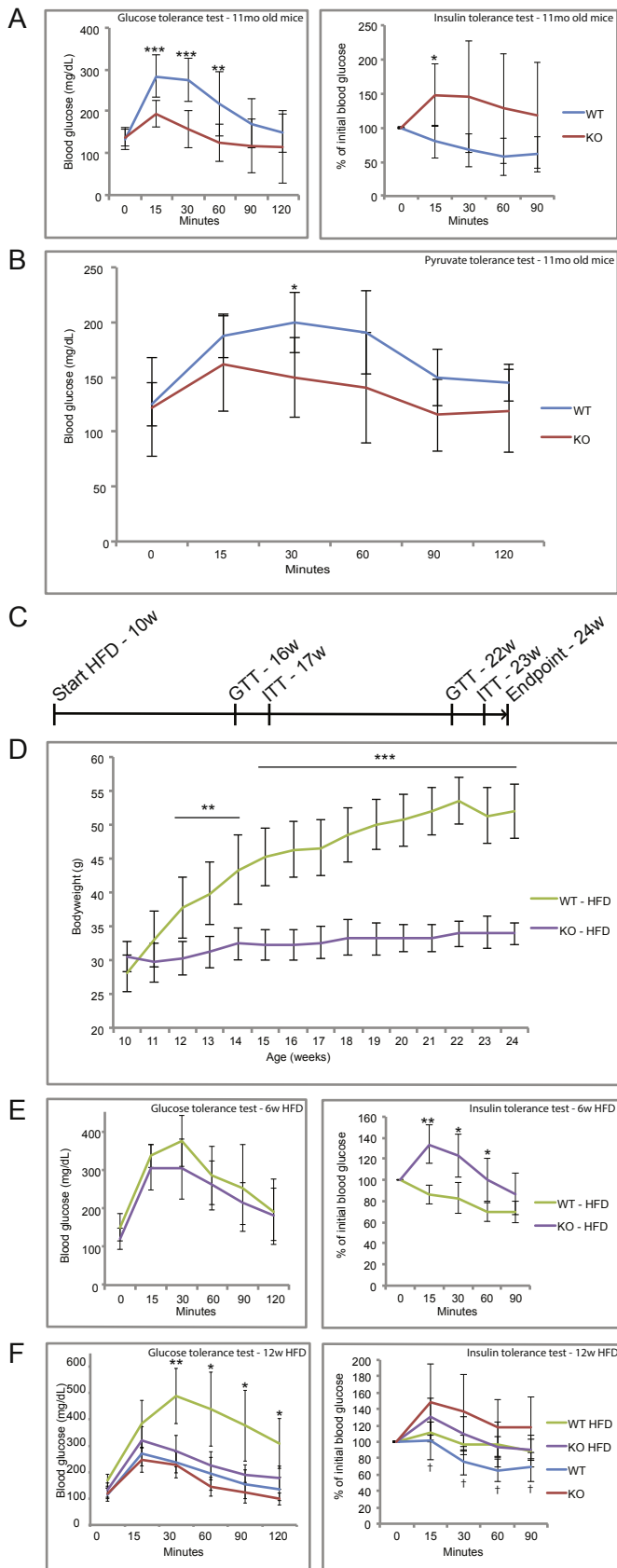


Fig. 2. AIRKO mice are protected from age- and HFD-related metabolic deficiencies. A) GTT and ITT performed on 11-month-old AIRKO mice and WT littermates. B) PTT performed on 11-month-old mice. C) Schematic showing the experimental design of the HFD study. D) Bodyweight measurements over time from the AIRKO and WT

2.4. Statistical analysis

All data points are represented as mean $\pm$ standard deviation and analyzed by unpaired two-tailed Student's *t*-test. Statistical significance is shown by * = *p*-value $\leq$ 0.05, ** = *p*-value $\leq$ 0.01, *** = *p*-value $\leq$ 0.001.

3. Results

3.1. AIRKO mouse characterization

AIRKO mice exhibit normal growth, as evidenced by unaltered bodyweight compared to WT controls (Fig. 1A). AIRKO mice do not develop gross obesity or severe lipodystrophy as a consequence of abrogated adipose IR activity. Upon dissection, AIRKO mice were found to have a reduction in adipose tissue mass whereas wild-type (WT) mice displayed normal adipose depots. To assess the effect of IR deletion in mature adipocytes we performed a glucose tolerance test (GTT) on 2-month-old mice. The AIRKO group displayed significantly higher blood glucose until 90 min after glucose administration (Fig. 1B). Insulin tolerance tests (ITT) revealed a significant reduction in insulin responsiveness (Fig. 1C). There is also significant fasting hyperglycemia after a 4-h fast before administration of the ITT (Fig. 1D). This presents a clear dysregulation of glucose and insulin metabolism, despite equivalent body weight.

3.2. The AIRKO mouse is protected from age- and diet-related metabolic deterioration

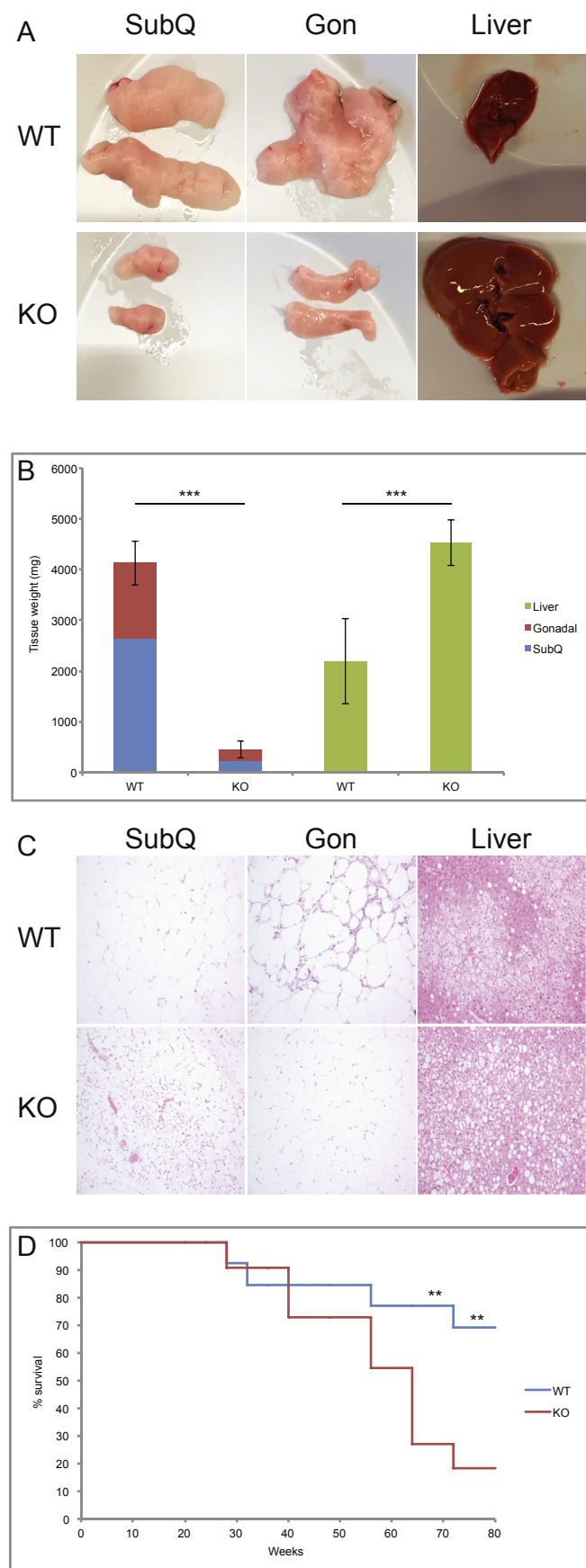
Mice acquire insulin resistance with age, leading to dysregulated glucose and lipid metabolism [14]. After finding reduced glucose tolerance and insulin sensitivity in 2-month-old mice, we decided to evaluate metabolic competency of aged AIRKO mice. A GTT of 11-month-old AIRKO mice revealed significantly higher glucose tolerance than the WT littermate control group, which acquired age-related glucose metabolism deficiencies (Fig. 2A). Despite this shift in glucose metabolism, the AIRKO group remains insulin resistant with a reduced response to insulin injection compared to WT controls (Fig. 2A). Fasting hyperglycemia in 11-month-old mice is consistent with findings in two-month-old mice (data not shown).

To investigate effects of adipose IR ablation on systemic non-adipose metabolic health, we studied hepatic gluconeogenesis using a pyruvate tolerance test (PTT). AIRKO mice display significantly less elevated blood glucose 30 min after the pyruvate bolus (Fig. 2B).

3.3. High-fat diet effects on AIRKO mouse metabolism

After assessing the effect of age-related insulin resistance on this mouse model, we subjected the AIRKO mouse to HFD, which is a standard mouse model of diet-induced obesity and associated metabolic effects. During the HFD regimen we performed GTTs and ITTs and regularly weighed the mice to monitor the metabolic health and the progression of obesity (Fig. 2C). AIRKO mice resisted weight gain while the WT littermates gained a statistically

groups on HFD. E) GTT and ITT performed on 16 week old mice after 6 weeks on HFD. F) GTT and ITT performed on 6 month old AIRKO and WT mice on chow or HFD as indicated. Mouse groups N = 10 WT and 6 KO for the age-study, N = 6 WT and 7 KO for the HFD study, N = 13 WT and 11 KO for the 6 month old chow cohort. Error bars, standard deviation. * = *p*-value $\leq$ 0.05, ** = *p*-value $\leq$ 0.01, *** = *p*-value $\leq$ 0.001, † = *p*-value $\leq$ 0.05 of WT versus other groups.



significant amount of mass (Fig. 2D). The WT mice suffer glucose intolerance as a consequence of HFD bringing them to parity with the AIRKO mouse after 6 weeks of HFD feeding (Fig. 2E). Despite this shift in relative glucose tolerance KO mice remain significantly insulin resistant after 7 weeks of HFD feeding, when compared to WT littermates (Fig. 2E). After 12 weeks of HFD administration, glucose sensitivity of AIRKO mice remained unchanged. In contrast, WT mouse glucose clearance worsened after 12 weeks of HFD, and was significantly lower than that of the AIRKO mice (Fig. 2F). After 13 weeks of the HFD regimen, the insulin sensitivity of WT mice has diminished to match that of AIRKO mice, regardless of the AIRKO dietary regimen (Fig. 2F).

3.4. Adipocyte IR expression is necessary for adipose tissue expansion and lifespan

Liver, subcutaneous adipose, and gonadal adipose tissue samples were collected and weighed at the end of the HFD regimen. Both fat depots were greatly reduced in mass in the AIRKO group, while the liver displayed clear enlargement (Fig. 3A). There was a 90% reduction in adipose tissue weight, and a doubling of the liver weight in AIRKO mice compared to WT controls (Fig. 3B). Cellular morphology corroborated this finding, as the adipocytes of the AIRKO mouse have almost no lipid droplets in the case of subcutaneous adipose, and reduced lipid droplet size in gonadal fat. Lipid accumulation is increased in AIRKO hepatocytes when compared to the WT sections (Fig. 3C).

In spite of the aforementioned enhanced glucose metabolism during aging relative to the control group, AIRKO mice have a significantly reduced median and maximum lifespan (Fig. 3D). This communicates a significant health burden conferred by the metabolic disadvantages of the AIRKO mouse, despite the buffering of age-related deterioration of insulin sensitivity and glucose tolerance.

3.5. Two month-old FIRKO mice are insulin insensitive

Previous reports have found metabolic and survival benefits from knocking out the IR in adipocytes (the FIRKO mouse) using the aP2 promoter-driven Cre recombinase [5,6]. We replicated the FIRKO mouse study to directly compare this model with the AIRKO mouse. FIRKO mouse fat pads were of insufficient mass for protein extraction, thereby confirming functional knockout and mirroring the phenotype of the AIRKO mouse. There is no bodyweight difference between FIRKO mice and WT littermates (Fig. 4A). In our hands FIRKO mice demonstrate hyperglycemia after an overnight fast, but are equally adept at clearing the injected glucose bolus as the control group when examined at two months old when subjected to a GTT (Fig. 4B). FIRKO mice are insulin resistant compared to WT littermates at two months of age as determined by ITT (Fig. 4B). When aged to six months, the FIRKO mouse no longer displays fasting hyperglycemia relative to WT and has normalized its insulin sensitivity to the level of the WT mouse (Fig. 4C). These phenotypes emulate the AIRKO mouse.

Fig. 3. IR knockout in adipocytes affects lipid accumulation and survival. A) Representative pictures of dissected subcutaneous (SubQ) and gonadal (Gon) adipose tissue as well as liver. B) Quantified weight of tissues shown in 3 A. C) Histology slides of dissected subcutaneous (SubQ) and gonadal (Gon) adipose tissue as well as liver. Images are taken at 20x zoom. D) Kaplan-Meier plot of the AIRKO mouse versus WT littermates. Mouse groups N = 6 WT and 7 KO for the HFD study, N = 13 WT and 11 KO for the start of the Kaplan-Meier plot. Error bars, standard deviation. * = p-value ≤ 0.05, ** = p-value ≤ 0.01, *** = p-value ≤ 0.001.

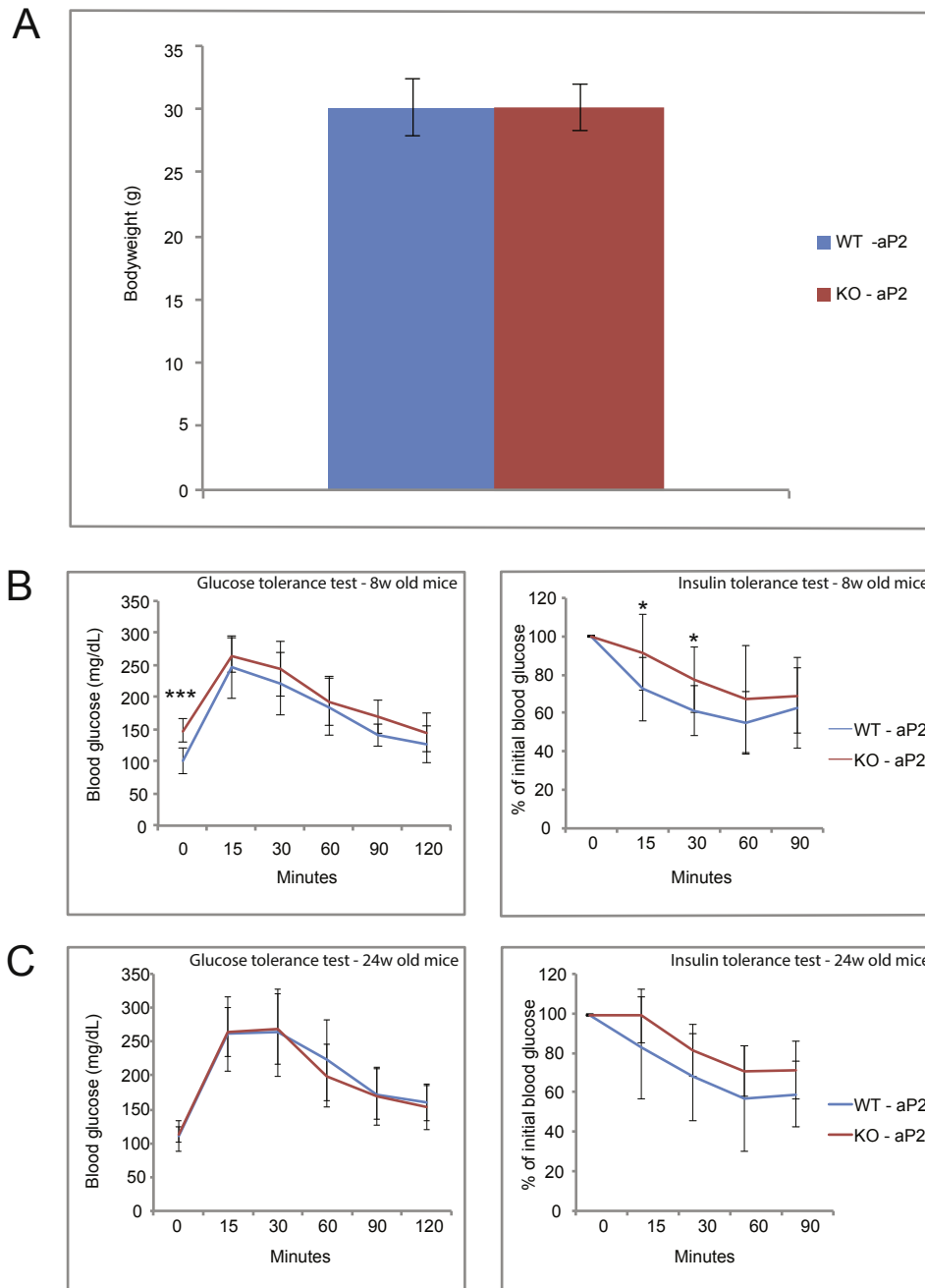


Fig. 4. The FIRKO mouse is insulin resistant at two months of age. A) Bodyweight of FIRKO and WT mice at 4 months old. B) GTT and ITT performed on 2-month-old mice. C) GTT and ITT performed on 6-month-old mice. Mouse groups N = 10 WT and 10 KO. Error bars, standard deviation. * = p-value ≤ 0.05 , ** = p-value ≤ 0.01 , *** = p-value ≤ 0.001 .

4. Discussion

In the present report we demonstrate the effects of adipocyte-specific deletion of the insulin receptor. This AIRKO model is protected from age-associated dysregulation of glucose metabolism, as well as obesity-induced insulin resistance. At two- and eleven-months of age AIRKO mice have identical blood glucose profiles during a GTT, exhibiting protection from age-related metabolic decline. AIRKO mice have identical weights on chow or HFD, as their adipose tissues do not expand in response to caloric excess. This suggests ablation of IR signaling in adipocytes prevents these mice from storing excess dietary fat in the adipose tissue. After HFD

treatment this deficiency leads to a 90% reduction in fat mass, while adipose is practically absent on a chow diet. This demonstrates the necessity of the insulin receptor for adipose tissue development and function.

Despite the relative protection from age- and diet-induced glucose intolerance, young AIRKO mice are glucose intolerant and insulin resistant compared to age-matched WT mice. AIRKO mouse insulin resistance at two months of age is equal in severity to the effects of 12 weeks of HFD-induced obesity or age-acquired insulin resistance on insulin insensitivity in WT mice. AIRKO mice also present with fasting hyperglycemia, divulging an obvious inadequacy in basal non-challenged glucose clearance. These

observations highlight the central role of adipose tissue insulin signaling in systemic glucose and insulin homeostasis. Disrupting this axis in AIRKO mice led to severe dysregulation of glucose homeostasis. These metabolic deficiencies resulted in a significant decrease in median and maximum lifespan in chow-fed AIRKO mice.

After 11 months of age, AIRKO mice cannot convert pyruvate to glucose during a PTT, indicating impairment of hepatic gluconeogenesis. This exhaustion of the liver's capabilities is likely due to increased hepatic triglyceride storage and resultant non-alcoholic fatty liver disease (NAFLD). The diagnosis of NAFLD is corroborated by the doubling of liver size of the AIRKO mice on HFD. Furthermore, triglyceride accumulation is increased in AIRKO livers (as evidenced by immunohistochemistry) as a consequence of decreased adiposity. This reveals that while abrogated insulin signaling in the adipose reduces the accumulation of fat in those depots, the liver now becomes the primary reservoir for triglyceride storage. The liver is a last resort to maintain lipid homeostasis in the absence of adipose tissue, but accumulation of excess hepatic lipids affects insulin insensitivity and diminishes metabolic functionality of the liver [15]. It is probable that skeletal muscle accumulates ectopic lipids in the AIRKO model as well, contributing to diminished insulin-stimulated glucose uptake.

Previous reports on the FIRKO model have shown beneficial health effects in these mice including extended lifespan and favorable effects on glucose clearance [5,6]. These results were directionally inconsistent with the phenotype of the AIRKO mouse. The FIRKO model exhibits more favorable glucose and insulin response profiles when compared with the deleterious AIRKO phenotype, but in our hands, the *aP2* promoter-driven IR knockout does not confer any positive effects on glucose clearance when compared to WT controls.

In the AIRKO model, insulin resistance is partly decoupled from glucose sensitivity. This implies there is some adipose function independent of systemic insulin resistance, which regulates glucose homeostasis. This adipose function is affected by HFD and age, resulting in significant glucose intolerance and insulin insensitivity in WT mice. The AIRKO mice are protected from both of these metabolic stressors. We speculate that this is due to the reduction in adipose depot size of the AIRKO mouse. In this model a reduction in adipocyte number leads to a decrease in adipokine secretion, which may mediate the systemic phenotypes we show. A number of adipokines exert control over glucose clearance in this context [16,17]. The delicate interplay between adipocyte insulin signaling, adipose depot mass, and aging remains to be further investigated. Inquiry into these interactions in the AIRKO model may identify therapeutic targets for the treatment of insulin resistance.

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Activation of IRF1 in Human Adipocytes Leads to Phenotypes Associated with Metabolic Disease

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SUMMARY

The striking rise of obesity-related metabolic disorders has focused attention on adipocytes as critical mediators of disease phenotypes. To better understand the role played by excess adipose in metabolic dysfunction it is crucial to decipher the transcriptional underpinnings of the low-grade adipose inflammation characteristic of diseases such as type 2 diabetes. Through employing a comparative transcriptomics approach, we identified *IRF1* as differentially regulated between primary and in vitro-derived genetically matched adipocytes. This suggests a role as a mediator of adipocyte inflammatory phenotypes, similar to its function in other tissues. Utilizing adipose-derived mesenchymal progenitors we subsequently demonstrated that expression of *IRF1* in adipocytes indeed contributes to upregulation of inflammatory processes, both in vitro and in vivo. This highlights *IRF1*'s relevance to obesity-related inflammation and the resultant metabolic dysregulation.

INTRODUCTION

Obesity is a disease in which the energy reserve stored in the adipose tissue has increased excessively to cause adverse health effects. Adipocytes play a critical role in the regulation of triglyceride storage, glucose homeostasis, and energy expenditure. Excess adipose can disrupt this regulation. Obesity-related metabolic diseases, including type 2 diabetes mellitus (T2DM), have reached epidemic proportions and have been recognized as a leading contributor to morbidity and premature mortality (Ogden et al., 2012). More than 85% of people diagnosed with T2DM are overweight or obese (ADA, 2012).

Adipocyte biology has garnered increased attention because of this. Chronic low-grade inflammation of adipose tissue has emerged as an important contributor to obesity-induced insulin resistance (Gregor and Hotamisligil, 2011). Inflammation of adipose tissue is thought to occur due to nutrient overload in adipocytes, activating inflammatory pathways and leading to the recruitment of immune cells. In the context of obesity, adipocytes are a critical source of inflammatory chemokines that contribute to metabolic disease.

Transcriptional profiling of adipocytes has identified several regulators of adipogenesis (transcription factors,

long non-coding RNAs, and microRNAs) (Urs et al., 2004). Unfortunately, few transcriptional regulators of adipose inflammation have been identified due to the in vitro nature of these studies. Therefore, the mechanistic underpinnings of how adipocytes contribute to metabolic diseases such as T2DM are underappreciated. To identify determinants of adipose inflammation, we transcriptionally profiled primary human adipocytes isolated from obese donors. We then compared expression data with in vitro-derived adipocytes. By focusing on differentially regulated transcription factors with a known function in immune response, we prioritized interferon regulatory factor 1 (IRF1). Subsequently we examined its role in adipose inflammation.

RESULTS

Comparative Expression Analysis of Primary versus In Vitro-Derived Adipocytes

For expression analysis of primary and in vitro-derived adipocytes, we obtained whole subcutaneous adipose tissue from two obese females (body mass index >30; 41 and 59 years old) undergoing elective abdominoplasty. Primary adipocytes were isolated from whole adipose tissue. We



verified the integrity of the adipocytes using fluorescence microscopy, showing the large unilocular lipid droplet typical of white adipocytes (Figure 1A). In addition, we isolated adipose stromovascular cells (ASCs) from one of the donors (59 years). The ASCs were subsequently differentiated into adipocytes at >90% efficiency (Figures 1B and S1A), and adipocyte-specific gene expression was measured (Figure S1B).

RNA was extracted from the above cell types and subjected to whole-genome microarray analysis. Hierarchical clustering accurately grouped replicates of adipocytes, ASCs, and ASC adipocytes, with significant expression differences between groups (Figure 1C). We identified 1,749 genes with >1.7-fold higher abundance in primary adipocytes versus ASC adipocytes at a 0.1% false discovery rate (FDR) (Table S1). Gene ontology enrichment analysis for the primary adipocyte-enriched genes was consistent with a phenotype of inflammation, with enriched terms including “response to type I interferon,” “immune response,” and “positive regulation of chemotaxis” (Table S2). Genes with increased expression in the aforementioned pathways reflect a spectrum of inflammatory mediators, including interferon-regulated transcription factors (*IRF1*, *IRF8*), chemokines (*CCL2/MCP1*, *CCL8*), cytokines (*IL6*, *IL8*), Toll-like receptors (*TLR1*, *TLR3*), and inflammatory components (*NLRP3*). This inflammatory signature was not observed in undifferentiated ASCs either.

To control for contribution of stromovascular fraction cells (SVFs) to the inflammatory expression signature we isolated the SVF pellet, as contaminating immune cell transcripts should be identified in this fraction as well. Of the 1,749 transcripts identified to be specifically upregulated in primary adipocytes compared with ASC adipocytes, 1,009 were more abundant in the primary adipocytes compared with the SVFs, providing a conservative estimate for the fraction of genes with an adipocyte origin (Table S3). This list of 1,009 genes includes all of the inflammatory mediators listed above. The highest expressed immunoregulatory transcription factor in primary compared with ASC adipocytes was *IRF1* (14.3-fold, $p = 7.50 \times 10^{-23}$). We sought to validate this result in two additional datasets. Comparing four additional primary adipocyte samples with three unmatched ASC adipocytes, we found a 33-fold upregulation of *IRF1* in the primary adipocytes (Figure 1D). To ascertain that increased *IRF1* expression is specific to obesity, we analyzed a publicly available dataset (GEO dataset GDS3602) comparing primary adipocytes from ten lean versus ten obese individuals, and found a 3.72-fold increase in *IRF1* expression in adipocytes from obese individuals (Figure 1E; Lee et al., 2005). A large portion of genes overexpressed in obese individuals from this dataset (1.7-fold, 0.1% FDR) (Table S4) overlaps with genes we found upregulated in primary adipocytes (Figure S1C). The list of overlap-

ping genes includes, but is not limited to, inflammatory genes such as the aforementioned *IRF1*, *CCL2*, *CCL8*, *IL6*, and *IL8* (Table S5).

Expression of *IRF1* in Adipocytes Activates the Immune Response

Utilizing lentivirus to express a doxycycline-inducible transgene, we devised a scheme to express *IRF1* in ASC adipocytes (*IRF1* adipocytes) (Figure 2A). After several days of doxycycline treatment we confirmed the expression of ectopic *IRF1* (Figure S2A). Chromatin immunoprecipitation qPCR (ChIP-qPCR) revealed that ectopic *IRF1* binds near genes that contain an *IRF1*-binding motif proximal to their promoter (*PARP14*, *IFIT3*, *ISG15*), and *IRF1* binding correlates with increased expression of these genes (Figure 2B). There is no expression difference or *IRF1* binding in genes without this motif (*OAS3*, *ZNF74*). When *IRF1* is not overexpressed, we do not see this effect (Figure S2B). In differentiated adipocytes, *IRF1* overexpression affected the mRNA levels of relatively few genes (138 upregulated, 80 downregulated, 1.7-fold, 0.1% FDR) (Figure 2C and Table S5). The majority of upregulated genes in *IRF1* adipocytes are associated with innate immunity, including major histocompatibility complex (MHC) class I receptors, interferon-responsive genes, and chemotactic cytokines. Immune response-associated genes found to be upregulated in *IRF1* adipocytes are also increased in primary adipocytes compared with ASC adipocytes. Furthermore, 103 of the 138 differentially regulated genes were previously reported as known interferon-responsive genes, confirming that we are observing a bona fide interferon-like response in *IRF1* adipocytes (Samarajiw et al., 2009). This leads us to conclude that ASC adipocytes expressing *IRF1* more closely resemble primary human obese adipocytes.

IRF1 Alters Metabolism and Lipid Droplet Composition of Adipocytes In Vitro

Experimental evidence shows that the interferon response pathway associates with altered adipocyte metabolism, as treatment of in vitro-derived adipocytes with interferon- γ induces insulin resistance (McGillicuddy et al., 2009). The known role of *IRF1* in the interferon response and the upregulation of inflammatory genes in *IRF* adipocytes led us to hypothesize that *IRF1* contributes to adipose inflammation. To further characterize *IRF1* activation, we performed a multiplex ELISA to determine levels of cytokines and adipokines released by *IRF1* adipocytes (Figures 3A and S2C). We found secretion of pro-inflammatory cytokines interleukin-6 (IL-6) and IL-8, as well as monocyte chemotactic protein 1 (MCP-1), were significantly elevated in *IRF1* adipocytes concomitant with the increase in RNA. We also found these genes to be upregulated in obese individuals (Figure S2D).

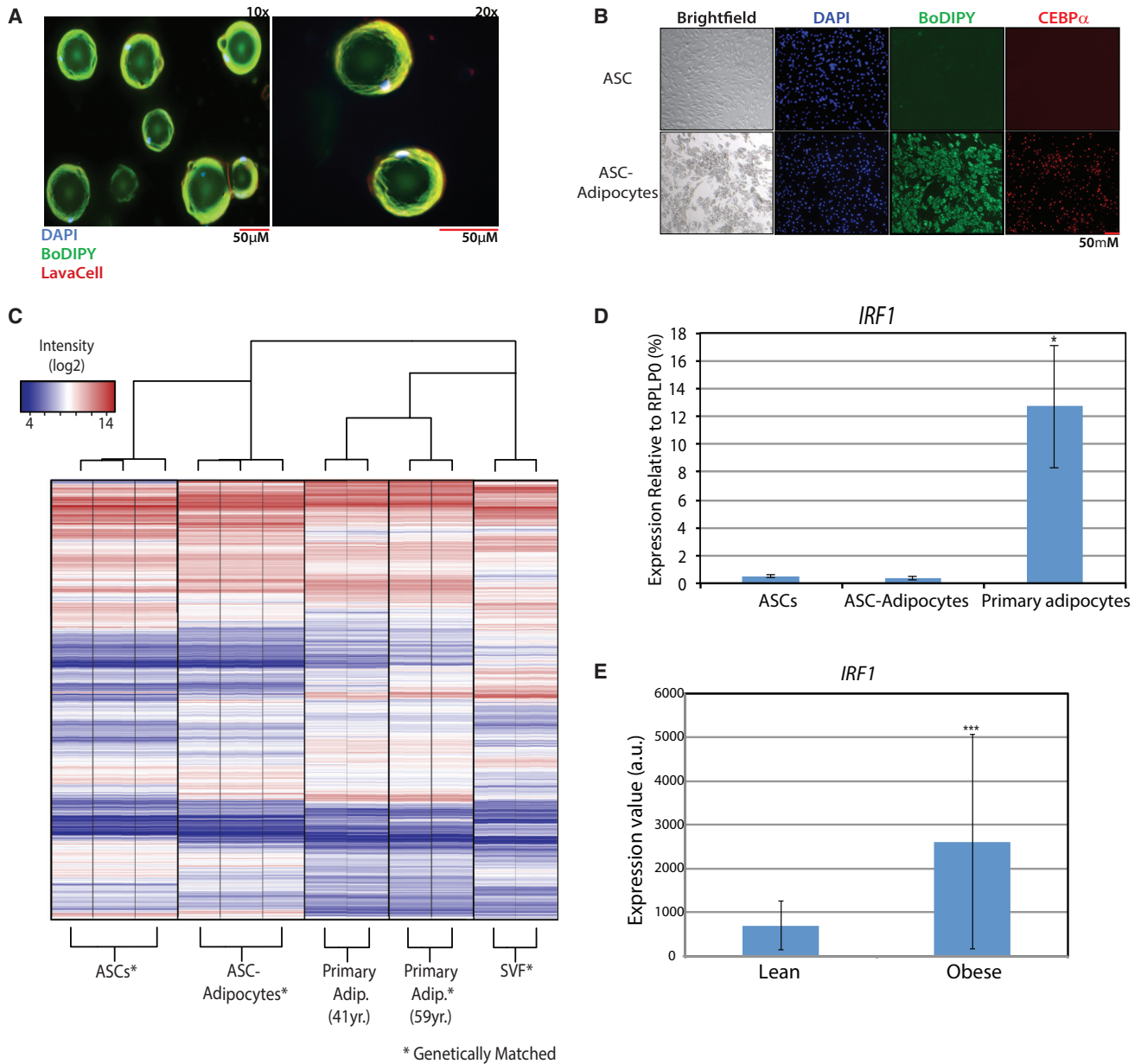


Figure 1. Primary and ASC-Adipocyte Integrity and Transcriptional Profiling

(A) Primary adipocytes isolated from whole adipose. Adipocytes were stained with DAPI to visualize the DNA (blue), BoDIPY to stain the lipid droplet (green), and LavaCell to stain the plasma membrane (red), and visualized by wide-field microscopy.

(B) ASCs were differentiated into adipocytes and stained with DAPI to visualize the DNA (blue), BoDIPY to stain lipids (green), and an antibody to visualize C/EBPα (red).

(C) Hierarchical clustering and heatmap representation of transcriptional profiles (mRNA) from ASCs, ASC adipocytes, and primary adipocytes. Probe sets are colored according to expression level.

(D) Gene expression of *IRF1* in ASCs (n = 3), ASC adipocytes (n = 3), and primary adipocytes (n = 4).

(E) Gene expression of *IRF1* in isolated subcutaneous adipocytes of lean (n = 10) and obese (n = 10) individuals.

For all graphs, error bars represent SD, experiments were performed in biological triplicates and statistically significant p values are denoted by asterisks (*p ≤ 0.05, **p ≤ 0.005).

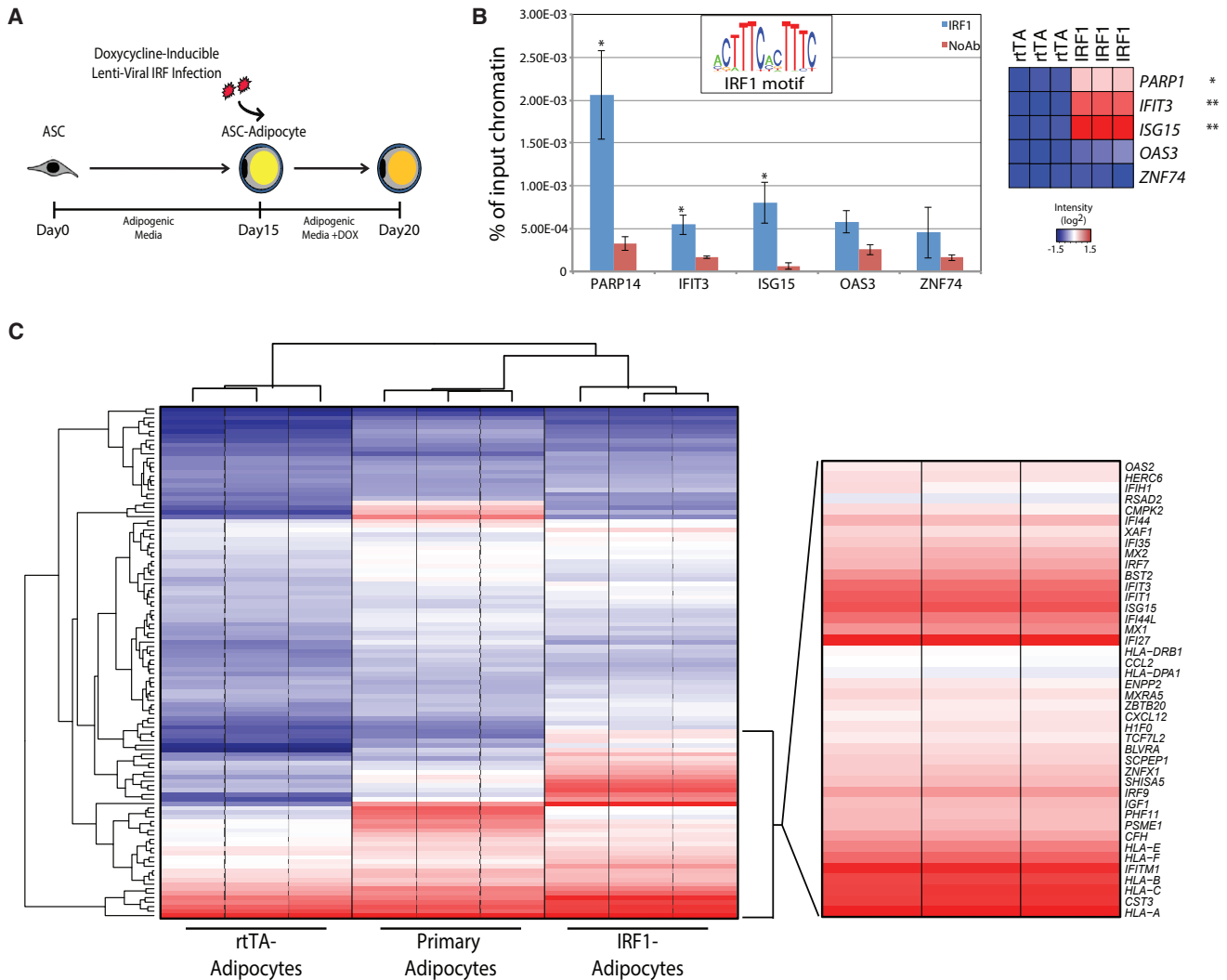


Figure 2. IRF1 Expression Leads to an Inflammatory Gene Signature

(A) Experimental scheme to ectopically express IRF1 in mature ASC adipocytes.

(B) ChIP-qPCR ($n = 3$) was performed for IRF1 at putative binding sites (H3K4me1 bound IRF1 motif) (*PARP14*, *IFIT3*, *ISG15*). *OAS3* and *ZNF74* represent a negative control site where IRF1 is not predicted to bind. Signals are expressed as percentage of total chromatin input. The heatmap represents expression levels of these genes in IRF1 and rTA adipocytes.

(C) Hierarchical clustering and heatmap representation of transcriptional profiles (mRNA) from rTA adipocytes, IRF1 adipocytes, and primary adipocytes. Probe sets are colored according to expression level. The highest expressed 30% of genes in IRF1 adipocytes have been expanded to highlight their inflammatory nature.

Error bars represent SD, experiments were performed in biological triplicates, and statistically significant p values are denoted by asterisks (* $p \leq 0.05$, ** $p \leq 0.005$).

Since obese adipocytes display reduced lipolysis in vivo, we performed a lipolysis assay to measure the ability of IRF1 adipocytes to catabolize intracellular lipids. In response to β -adrenergic stimulation, IRF1 adipocytes were unable to increase triglyceride hydrolysis, unlike control cells (Figures 3B and S2E). To assess insulin sensitivity, we performed a glucose uptake assay. Expression of *IRF1* abrogates insulin-mediated glucose uptake in ASC adipo-

cytes, consistent with the increase in pro-inflammatory signaling and altered lipid metabolism (Figure 3C).

Unlike their in vivo counterparts, in vitro-derived adipocytes typically display multilocular lipid droplets. After several days of *IRF1* expression, ASC adipocytes demonstrate a transition from a multilocular to unilocular state and the average size of the largest lipid droplet increases significantly (Figure 3D). The total intracellular triglyceride

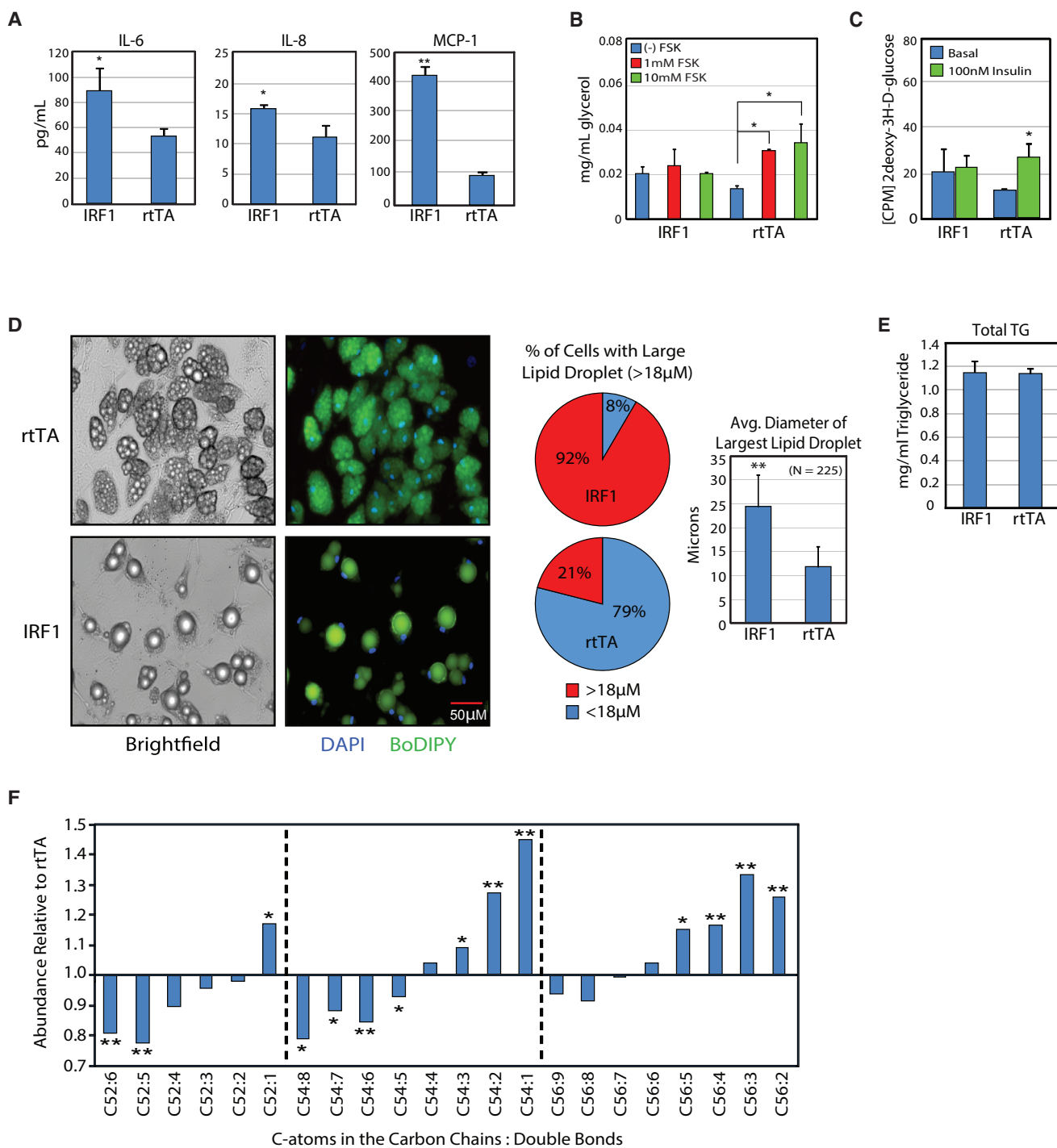


Figure 3. IRF1 Expression Impairs Adipocyte Metabolism and Affects Lipid Composition

(A) Multiplex ELISA assay was utilized to measure cytokines secreted into medium by IRF1 adipocytes compared with control adipocytes. (B) Glycerol release into the medium in response to forskolin (FSK)-activated lipolysis was measured in IRF1 and rtTA adipocytes. (C) Radiolabeled glucose uptake was measured in response to insulin. (D) After 5 days of IRF1 expression, IRF1 adipocytes become unilocular. Lipid droplet size was measured after staining with the lipid dye BoDIPY (green) and the nuclear dye DAPI (blue). CPM, counts per minute.

(legend continued on next page)



(TG) content in IRF1 adipocytes is similar to that of ASC adipocytes, indicating no lipid uptake or synthesis phenotype (Figure 3E). These data suggest that IRF1 has a specific effect on lipid droplet coalescence. Next we used tandem mass spectroscopy to assess lipid composition. We found that intracellular TGs in IRF1 adipocytes exhibit a greater degree of saturation, with fewer double bonds ($p = 7 \times 10^{-6}$) and longer carbon chains ($p = 8 \times 10^{-6}$) (Figures 3F and S2F).

IRF1 Expression in Adipose Leads to Localized Inflammation In Vivo

IRF1-adipocyte phenotypes in vitro are consistent with those reported for inflamed primary adipose tissue; hence, we tested whether IRF1 also contributes to adipose inflammation in vivo. We transfected the adipogenic mouse cell line 3T3-F442A with a human *IRF1* (*hIRF1*) overexpression construct or control (Figure S3A). These cells were injected subcutaneously onto the flanks of nude mice. After 6 weeks of engraftment, ectopic fat pads were excised and assayed for inflammatory phenotypes (Figure 4A). We observed a significant increase in the number of macrophages localizing to implants expressing *IRF1* compared with control implants or autologous white adipose tissue (WAT) (Figures 4B, 3C, S3B, and S3C). IRF1 implants exhibited increased expression of MHC class II and MCP-1 compared with autologous WAT, as well as an increase in the macrophage-specific gene *F4/80*, in line with the immunostaining shown above (Figure 4D). Inexplicably, we did find noticeable differences in gene expression between autologous WAT of *hIRF1* and *rtTA* (reverse tetracycline-controlled transactivator) mice despite using biological triplicates. While this is worrying, there is consistency with the staining data. Altogether these data are consistent with both the transcriptional analysis of primary adipocytes and in vitro assays performed in ASC adipocytes, and imply that *IRF1* contributes to adipose inflammation.

DISCUSSION

The mechanistic underpinnings of obesity's contribution to chronic adipose inflammation are poorly understood. Few transcriptional regulators have been identified that activate adipose inflammatory cascades in vivo. Progress is impeded by the lack of direct experimentation on primary adipocytes and reliance on in vitro systems that

cannot recapitulate obesity, endocrine regulation, and immune system interactions. However, previous studies have implicated the major inflammatory transcription factors nuclear factor κ B and activator protein 1 as possible mediators of adipose inflammation associated with obesity, but the mechanism for activation is unclear (Suganami et al., 2007; Osborn and Olefsky, 2012). This study found several inflammatory mediators differentially expressed in primary versus differentiated adipocytes, most notably *IRF1*. This result was verified in an independent dataset showing an increase of *IRF1* in obese individuals when compared with lean individuals. *IRF1* has been identified as a transcriptional activator of type I interferon gene activation in response to viral infection (Harada et al., 1989). Additionally it has been observed that *IRF4* represses early stages of adipogenesis (Eguchi et al., 2008) and modulates adipocyte lipid handling in vitro (Eguchi et al., 2011), suggesting a significant role for IRFs in adipose biology. However, there has been no study of *IRF1* in human adipocytes. Expression of *IRF1* in ASC adipocytes is sufficient to activate genes in the interferon response and inflammation pathways. Despite few transcriptional changes, *IRF1* overexpression leads to phenotypes associated with metabolic disease, including insulin resistance and attenuated lipolysis. Further supporting its role in adipose inflammation, expression of *IRF1* in 3T3-derived mouse adipose in vivo increases macrophage infiltration. We also demonstrate elevated MCP-1, which leads to inflammation and is associated with obesity, insulin resistance, and altered adipocyte metabolism (Kanda et al., 2006; Kim et al., 2006).

Lipidomic profiling of human subjects has revealed that the level of circulating saturated TGs is strongly correlated with insulin resistance and is a predictor of T2DM development (Rhee et al., 2011; Würtz et al., 2012). One striking phenotype of IRF adipocytes is the change in length and saturation level of TGs. The finding that expression of an immunoregulatory transcription factor leads to changes in composition of lipid droplet TGs is surprising. This finding in conjunction with our lipidomics data suggests that inflammation triggers production of more highly saturated fatty acids, in turn driving ER stress and further inflammation in a feedback loop (Wang et al., 2006). IRF1 adipocytes also acquire a unilocular lipid droplet, characteristic of primary adipocytes but absent in all in vitro models. Longer-chain, saturated TGs in IRF1 adipocytes could increase hydrophobicity of the intracellular lipids, potentiating the observed coalescence of lipids into a single large droplet.

(E) Total intracellular triglycerides (TG) were measured in IRF1 and *rtTA* adipocytes.

(F) Tandem mass spectrometric profiles of triglyceride composition for both IRF1 and *rtTA* adipocytes.

Error bars represent SD, experiments were performed in biological triplicates, and statistically significant *p* values are denoted by asterisks (**p* ≤ 0.05, ***p* ≤ 0.005). *n* = 3 for each experiment.

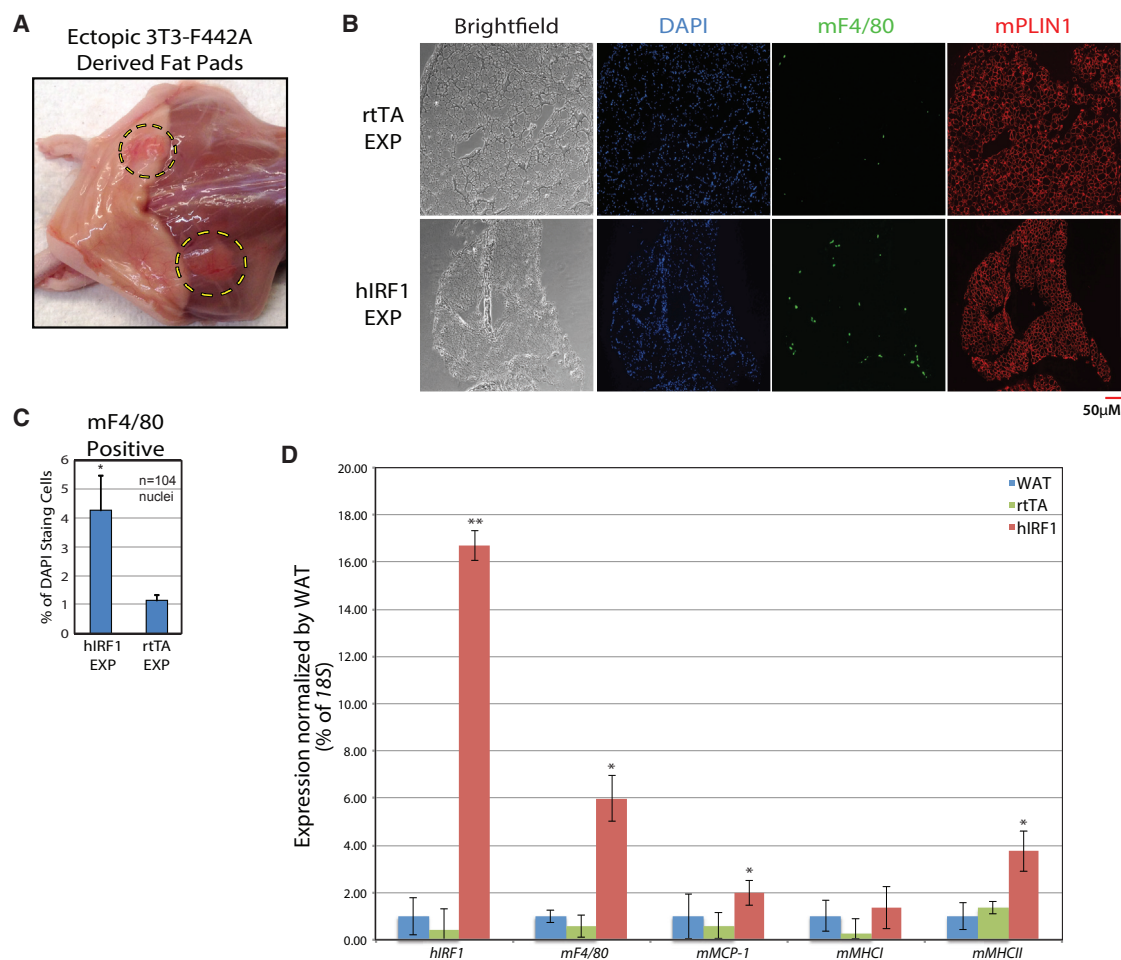


Figure 4. IRF1 Leads to Increased Inflammation In Vivo

(A) 3T3-F442A cells were transduced with either rtTA alone, or human IRF1 (hIRF1) containing lentiviral particles, and were injected into 6-week-old nude mice ($n = 4$). Six weeks after injection, ectopic subcutaneous fat pads (dashed circles) were excised.

(B) Ectopic fat explants (EXP) were stained with DAPI (blue) and probed with α -F4/80 (green) and α -PLIN1 (red).

(C) F4/80-positive cells were counted relative to DAPI staining foci in hIRF1 explants versus rtTA explants and autologous WAT (see Figure S3C). A minimum of 10^4 DAPI foci were counted for each experiment.

(D) qPCR was used to assess expression of hIRF1 and inflammation-associated mouse genes in both explants and autologous WAT ($n = 3$). Error bars represent SD, experiments were performed in biological triplicates, and statistically significant p values are denoted by asterisks ($*p \leq 0.05$, $**p \leq 0.005$).

Our data suggest that *IRF1* expression in human adipocytes contributes to adipose inflammation and insulin resistance.

EXPERIMENTAL PROCEDURES

Primary Adipocyte Isolation and ASC Cell Culture/Differentiation

Primary human adipocytes were obtained from discarded adipose tissue from two female elective abdominoplasty patients. Whole adipose was chopped up and digested with Liberase Blendzyme (Roche) for 1 hr at 37°C , forced through a $250\text{-}\mu\text{m}$ filter, and centrifuged. The floating adipocyte fraction was washed with PBS and

re-collected. The stromovascular cell pellet was washed twice with PBS and plated onto gelatin-coated plates in ASC growth medium (DMEM, 10% fetal bovine serum [FBS], 1% penicillin-streptomycin, and 2.5 ng/mL basic fibroblast growth factor [Aldevron]). ASCs were brought to homogeneity by passaging and when $\sim 95\%$ confluent, adipocyte medium (DMEM, 10% FBS, 1% penicillin-streptomycin, $0.1\text{ }\mu\text{M}$ dexamethasone, $10\text{ }\mu\text{g/mL}$ insulin, $0.5\text{ }\mu\text{M}$ rosiglitazone, $250\text{ }\mu\text{M}$ 3-isobutyl-1-methylxanthine [IBMX]) was added for 5 days. On day 6, ASCs were switched to adipocyte medium without IBMX for 14 additional days.

RNA Extraction/qRT-PCR/Transcriptional Profiling

Total RNA from all cell preparations was extracted using TRIzol (Invitrogen). RNA quality was determined using an RNA nanochip



on a Bioanalyzer (Agilent Technologies). RNA was converted to cDNA using the Superscript First-Strand III kit (Invitrogen) and qRT-PCR was carried out using a Realplex Mastercycler (Eppendorf) with Quantifast-SYBR Green Mastermix (Qiagen). Primer sets are listed in [supplemental Experimental Procedures](#).

RNA samples were processed by Asuragen and microarray analysis was performed on the Affymetrix Human Genome U133 Plus 2.0 Array. Arrays were hybridized overnight at 50°C, washed, stained, and scanned on a GeneChip Scanner 3000. Microarray data was analyzed using R/Bioconductor (<http://www.bioconductor.org>). Raw data were normalized by robust multi-array averaging (Bolstad et al., 2003) using a custom Chip Description File from the Michigan Microarray Lab (<http://brainarray.mbnl.med.umich.edu>, version 13). Primary adipocyte RNA is from Zenbio, cat. #RNAmi-Q10-1, RNAmi-Q10-2, and RNA-Q10-3. GEO data were taken from Lee et al. (2005), dataset record GEO: GDS3602.

Microarray Gene and Sample Clustering

To select a minimally biased set of genes for hierarchical clustering, we used the R package limma (Smyth, 2005) to test for differential expression across all ten pairwise comparisons between biologically distinct samples: primary adipocytes (59 and 41 years of age), ASCs (59 years), ASC adipocytes (59 years), and the pelleted SVF from disassociated adipose tissue (59 years). Hierarchical clustering was performed on the 2,489 probe sets with five or more significantly different pairwise comparisons (FDR = 0.001). The hmap function in the seriation R package (Hahsler et al., 2008) was used to generate the heatmap.

Production of Inducible Transgenes/Lentivirus Transduction

Doxycycline-inducible lentiviral Gateway (Invitrogen) vector containing *IRF1* was constructed by PCR amplification from verified cDNA clones (GenBank: NM_002198) (GeneCopoeia), and standard TOPO cloning (Invitrogen) techniques were used to generate entry clones. The *IRF1* construct was transduced via calcium chloride transfection into HEK293T cells as previously described (al Yacoub et al., 2007). Media containing viral supernatant was collected, filtered, and used to infect ASC adipocytes. Infection efficiency was measured as compared by co-infection with a GFP lentiviral construct. Expression of *IRF1* was induced by addition of doxycycline (1 µg/mL final) to the adipogenic medium.

ChIP

ASC adipocytes were crosslinked in 1% formaldehyde and quenched with glycine (200 mM final). Cells were washed several times in PBS. ChIP-qPCR was performed with an antibody against *IRF1* (Santa Cruz Biotechnology, sc-497) as previously described (Camahort et al., 2007). All ChIPs were graphed as percentage of total chromatin input. qPCR was performed as described above.

Protein Normalization

Protein concentration used to normalize other assays was measured using the Bradford protein assay (Bio-Rad).

Metabolic Assays

For glucose uptake, after 5 days of *IRF1* expression ASC adipocytes were serum starved in low-glucose DMEM containing 0.2% BSA overnight. Cells were incubated in KRH buffer (120 mM NaCl, 5 mM KCl, 1 mM MgSO₄, 0.3 mM CaCl₂ and 10 mM HEPES [pH 7.4]) in the presence or absence of 100 nM insulin for 30 min at 37°C. Non-specific uptake was measured in the presence of 10 µM cytochalasin B. Glucose uptake was measured by incubating cells with 0.5 µCi/mL⁻¹ 2-deoxy-D-[³H]glucose (Perkin-Elmer) for 5 min at 37°C.

For glycerol release, after 5 days of *IRF1* expression ASC-adipocyte cells were starved in DMEM with 1% FBS for 1 hr and then incubated in Hank's balanced salt solution with 2% fatty acid-free BSA with forskolin for 1 hr (0 µM, 1 µM, and 10 µM). The incubation medium was collected for glycerol measurement using a colorimetric assay kit (Sigma).

All assays were performed in biological triplicates and normalized to protein.

Multiplex ELISA Assay

Medium was collected 5 days after *IRF1* expression and ELISA was performed on a Luminex 200 analyzer using the Human Adipocyte Multiplex Panel (Millipore, HADCYMAG-61K) as per manufacturer's instructions. The assay was performed in biological triplicate and normalized to protein.

Triglyceride Analysis/Lipidomics

Total TG levels were measured using a colorimetric assay kit (Cayman Chemicals). Protein concentration was used to normalize intracellular TG content. For lipidomics, after 5 days of transgene expression ASC adipocytes were washed with fresh medium. Ice-cold isopropanol was added, and cells were scraped and collected into cold tubes. Extracts were incubated for 1 hr at 4°C, then vortexed and centrifuged at 2,300 × g for 10 min. The supernatant was used for mass spectroscopic analysis. All data were acquired using a Sciex 4000 QTRAP mass spectrometer as previously described (Rhee et al., 2011). MultiQuant software (version 1.1; Applied Biosystems/Sciex) was used for automated peak integration, and peaks were manually reviewed for quality of integration. Internal standard peak areas were monitored for quality control and used to normalize analyte peak areas.

For the transduced ASCs we averaged six replicates for metabolite abundance of 44 TG species and computed an abundance ratio of the two (*IRF1*/rtTA). We built a regression model, predicting the abundance ratio using number of double bonds and total number of carbon atoms as variables as well as an interaction term to assess whether the relationship with number of double bonds varies with carbon number. Statistical significance of regression coefficients was assessed using the F test.

Transplantation

Differentiated 3T3-F442A preadipocytes were transduced with *hIRF1* or *rtTA* lentivirus at day 7 and injected subcutaneously on the backs of 6-week-old female NCr nude mice (n = 4, Taconic). The cells developed into mature adipocytes over the next 6 weeks. Mice were euthanized to harvest fat pads which were fixed,



embedded in paraffin, and stained after sectioning. Harvard University Animal Care and Use Committee approved all animal procedures.

Immunostaining

The following antibodies and dilutions were used: α -CEBPA 1:100 (Santa Cruz, sc-61), α -PLIN1 1:200 (Sigma, P1873), and α -F4/80 1:200 (eBioscience, 14-4801-81). Lipid droplets were stained using BoDIPY neutral lipid dye (Thermo Fisher, D-3922), cell membranes using LavaCell dye (Active Motif, 15004), and cell nuclei with DAPI. Images were acquired using a Nikon Eclipse Ti-S microscope. Both NIS-Elements and ImageJ software packages were used for image analysis.

ACCESSION NUMBERS

All microarray data were deposited in the GEO under GEO: GSE96062.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and five tables and can be found with this article online at <http://dx.doi.org/10.1016/j.stemcr.2017.03.014>.

AUTHOR CONTRIBUTIONS

M.F. and R.C. performed the research and wrote the manuscript. Y.-K.L. performed the research with support of F.X. R.E.G. and E.P.R. performed the lipidomics. R.C.D. carried out the bioinformatics analysis. C.A.C. designed the study, supported the finance, and approved the final manuscript.

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Asialoglycoprotein receptor 1 is a specific cell-surface marker for isolating hepatocytes derived from human pluripotent stem cells

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ABSTRACT

Hepatocyte-like cells (HLCs) are derived from human pluripotent stem cells (hPSCs) *in vitro*, but differentiation protocols commonly give rise to a heterogeneous mixture of cells. This variability confounds the evaluation of *in vitro* functional assays performed using HLCs. Increased differentiation efficiency and more accurate approximation of the *in vivo* hepatocyte gene expression profile would improve the utility of hPSCs. Towards this goal, we demonstrate the purification of a subpopulation of functional HLCs using the hepatocyte surface marker asialoglycoprotein receptor 1 (ASGR1). We analyzed the expression profile of ASGR1-positive cells by microarray, and tested their ability to perform mature hepatocyte functions (albumin and urea secretion, cytochrome activity). By these measures, ASGR1-positive HLCs are enriched for the gene expression profile and functional characteristics of primary hepatocytes compared with unsorted HLCs. We have demonstrated that ASGR1-positive sorting isolates a functional subpopulation of HLCs from among the heterogeneous cellular population produced by directed differentiation.

KEY WORDS: Hepatocytes, Human pluripotent stem cells, FACS, MACS, Transcriptomics

INTRODUCTION

The hepatocyte mediates many liver functions by carrying out a multitude of activities at the cellular level. Despite the regenerative capacity of the liver *in vivo*, primary human hepatocytes (PHHs) are not viable *in vitro*, and they are in limited supply. Although micropatterned co-culture can extend the viability of PHHs *ex vivo* (Khetani and Bhatia, 2008), there is a substantial need for a renewable source of human hepatocytes for *in vitro* studies and the development of cell-based therapies. Human pluripotent stem cells (hPSCs) are a promising source of these cells (Schwartz et al., 2014).

There are well-established methods for the directed differentiation of hepatocytes from hPSCs using defined media and feeder-free culture conditions (Mallanna and Duncan, 2013). These protocols can be used to produce hepatocytes from hPSCs, generating a cellular population at least 70% positive for the hepatocyte-specific marker albumin. These cells also express other hepatocyte-specific genes and perform many of the hallmark

cellular functions of hepatocytes, such as cytochrome activity and apolipoprotein secretion. However, hPSC-derived hepatocytes are not equivalent to primary adult human hepatocytes and are more accurately considered ‘hepatocyte-like cells’ (HLCs). Unlike adult hepatocytes, HLCs typically retain expression of the fetal hepatocyte marker alpha fetoprotein (AFP) and fall considerably short of mature hepatocytes in terms of quantifiable functional capabilities, such as albumin secretion and drug detoxification.

Substantial obstacles must be overcome before advanced disease modeling studies can be attempted with HLCs. One notable hurdle is the variability and inefficiency of differentiation (Bock et al., 2011; Osafune et al., 2008; Takayama et al., 2014). Evidence suggests that this characteristic variability stems from inherent differences in hPSC lines (Kajiwara et al., 2012). This problem poses a challenge for *in vitro* modeling of subtle phenotypes, as well as phenotypes that could be confounded by incomplete or inaccurate differentiation. Here we describe the validation of a strategy for the prospective isolation of HLCs differentiated from a variety of hPSC lines based on the expression of a liver-specific cell surface protein, ASGR1. ASGR1 has long been recognized as a hepatic surface marker (Ashwell and Morell, 1974; Schwartz et al., 1981) and has been used to identify circulating hepatocellular carcinoma cells (Li et al., 2014), purify hPSC-derived HLCs (Basma et al., 2009) and to demonstrate the efficiency of HLC differentiation from hPSCs (Takayama et al., 2014). Whereas the utility of ASGR1 as a marker of hepatocyte identity is well established, the subpopulation of cells expressing ASGR1 in hPSC-derived HLCs has not been rigorously studied on the transcriptional level. To improve our understanding of the ASGR1-positive subpopulation of hPSC-derived HLCs and in the interest of developing a strategy for the purification of functional HLCs, we extensively characterized ASGR1-positive cells. ASGR1 marks a subset of albumin-positive HLCs, which are more similar than unpurified cells to mature hepatocytes. Furthermore, we show that ASGR1-enriched HLCs can be replated for further functional analysis, while retaining hepatocyte marker expression and cellular functions for up to 72 hours after sorting. These purification strategies increase the utility of hPSC-derived HLCs by enabling the isolation of a homogeneous population of hepatocytes for functional studies.

RESULTS AND DISCUSSION

Directed differentiation of HLCs

Depending on the hPSC line used and other experimental variables, differentiation generally results in a mixture of HLCs (the desired cell type) and a variable number of other cell types (Fig. 1A). The specific composition of mixed HLC differentiation cultures has not been investigated. Our laboratory has developed an optimized HLC-directed differentiation protocol based on established methods (Pagliuca et al., 2014; Si-Tayeb et al., 2010) with modest modifications.

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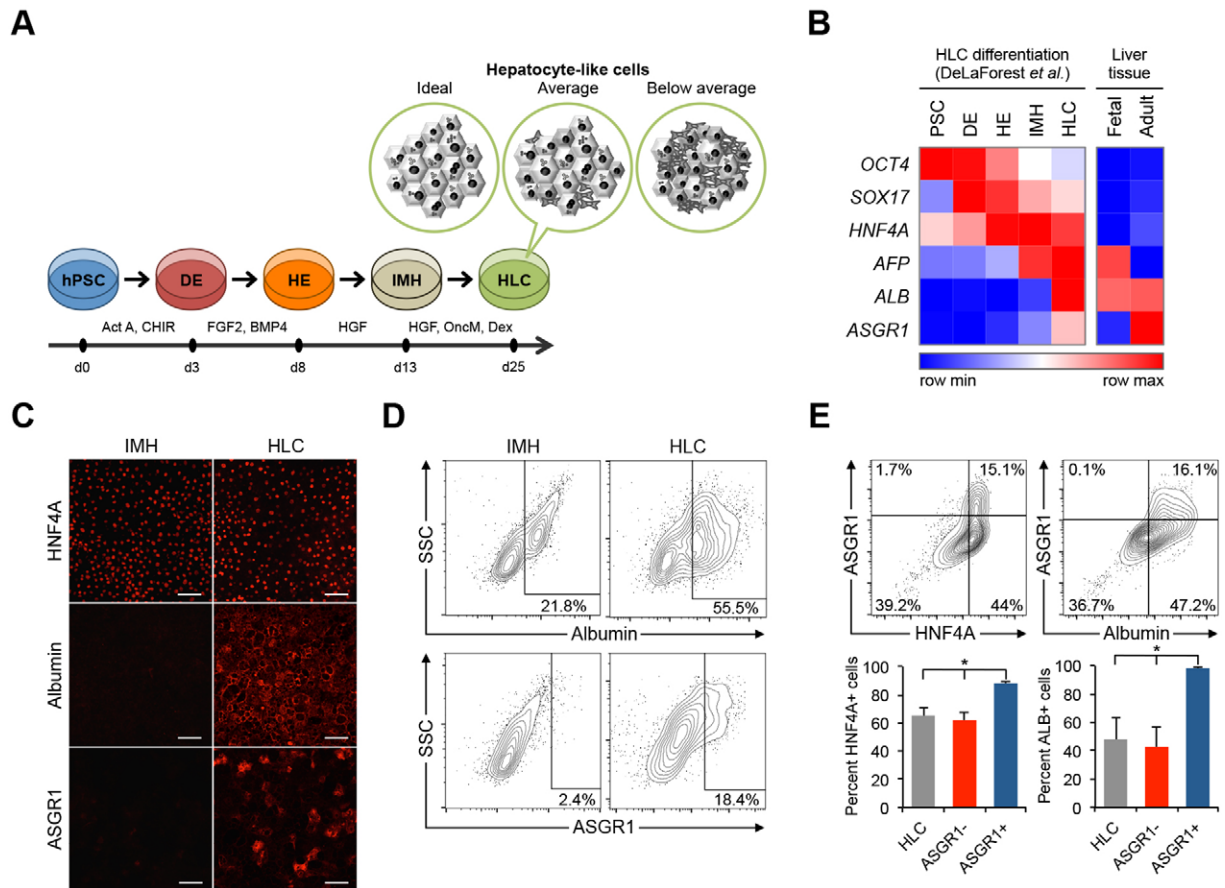


Fig. 1. Directed differentiation of hPSCs to hepatocyte-like cells (HLCs). (A) Overview of optimized protocol for directed differentiation from hPSCs to HLCs. Non-hepatic cell types contaminate the cell culture in suboptimal differentiation conditions. (B) Heatmap showing gene expression level of representative markers during each stage of HLC differentiation *in vitro* and in normal liver tissue *in vivo* from published microarray expression data (DeLaForest et al., 2011; Su et al., 2004). Expression values are row normalized; red denotes higher than average expression and blue denotes lower than average expression for each gene. (C) Confocal microscopy images of immunocytochemical staining after the third (IMH) and fourth (HLC) differentiation stages. Scale bars: 100 μm. (D) Representative flow cytometry analyses of ALB and ASGR1 expression at the IMH and MH differentiation stages. Similar patterns were observed in multiple independent differentiations (Fig. S1D). (E) Representative flow cytometry analyses showing co-expression of ASGR1 with HNF4α or ALB. Results of two independent differentiations per analysis are shown in the accompanying bar charts. Error bars represent s.e.m. **P* < 0.05, Student's *t*-test.

We analyzed published gene expression data from human tissues as well as from HLC differentiation of hPSCs and found that *ASGR1* is expressed in adult liver tissue, is not expressed or is present at an extremely low level in fetal liver, and is expressed most highly during HLC differentiation after the final differentiation stage – the ‘HLC’ stage (Fig. 1B). We confirmed this expression pattern during HLC differentiation by immunocytochemistry (Fig. 1C) and flow cytometry (Fig. 1D,E), which indicate that albumin (ALB) and ASGR1 are expressed at a very low level by a minority of cells at the end of the immature hepatocyte stage (IMH) and that they are both far more prevalent at the HLC stage of differentiation.

ASGR1 marks a subset of HLCs

Using our HLC differentiation protocol we found that ASGR1 is present in a small number of cells after the third differentiation stage (the IMH stage), and is more prevalent at the final stage of differentiation (Fig. 1D). This is in contrast to the expression pattern of the secreted protein ALB (a marker of functional hepatocytes), which is expressed at the IMH stage as well as the HLC stage (Fig. 1D).

The percentage of ASGR1-positive cells is almost always lower than that of ALB-positive cells in published reports as well as in our differentiations (Takayama et al., 2014). To confirm that ASGR1-

positive cells present at the end of HLC differentiation are in fact a subset of HLCs, we performed intracellular flow cytometry by co-staining for ASGR1 and other markers. We found that ASGR1-positive cells occur within a subpopulation of differentiated cells that express the hepatocyte lineage marker hepatocyte nuclear factor 4 alpha (HNF4α), as well as ALB (Fig. 1E).

Enrichment of differentiated hepatocytes based on ASGR1 surface expression

We next investigated the utility of ASGR1 for prospective hepatocyte isolation. We differentiated multiple human embryonic stem cell (hESC) and human induced pluripotent stem cell (hiPSC) lines representing a range of HLC differentiation propensities and characterized the expression of hepatocyte markers among cells positive for surface ASGR1. Fluorescence-activated cell sorting (FACS) analysis following HLC differentiation of four hPSC lines showed that a large proportion of surface ASGR1-positive cells were also ALB positive, even when the overall differentiation efficiency was extremely low (2.97% ASGR1-positive cells, Fig. 2A, summarized in Fig. 2B). Similar results were obtained when the expression of alpha-1 antitrypsin (AAT; SERPINA1 – Human Gene Nomenclature Committee), an additional marker of functional hepatocytes, was assessed (Fig. S2B).

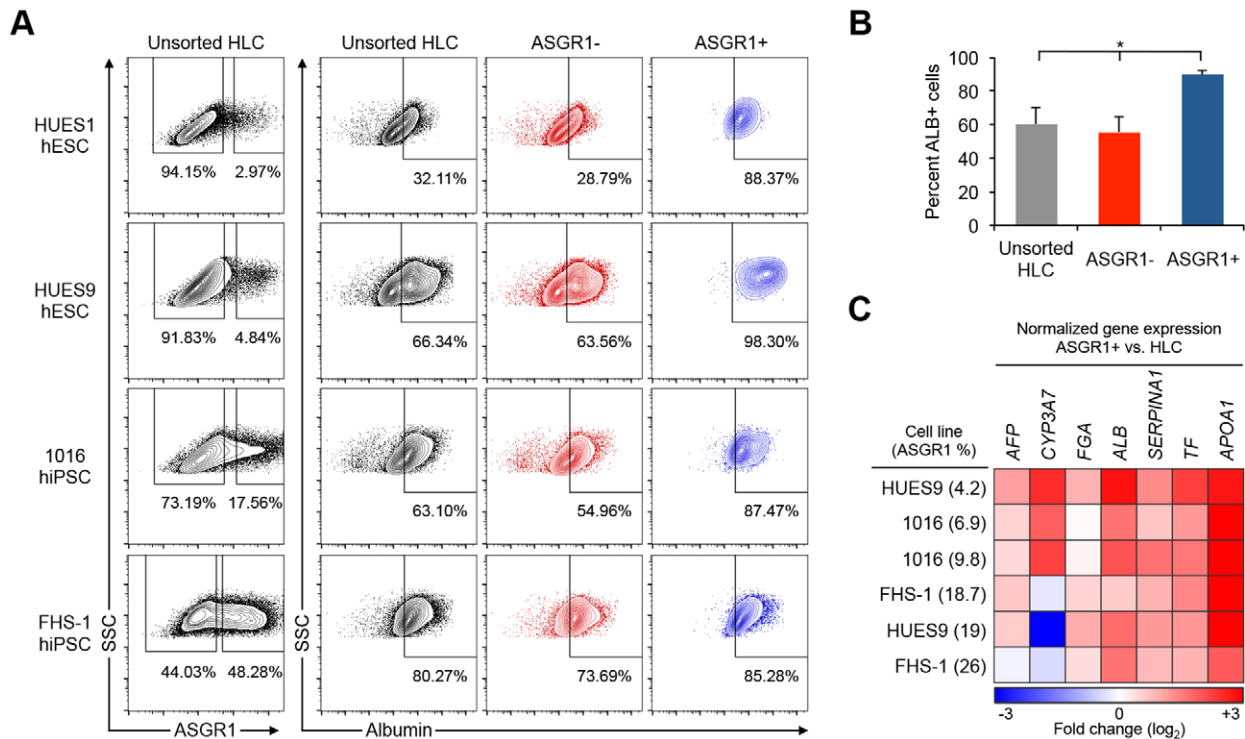


Fig. 2. Enrichment of hepatocytes from HLC differentiation cultures by surface ASGR1 FACS. (A) Four different hPSC lines were differentiated to HLCs. The percentage of cells expressing the hepatocyte marker ALB among unsorted HLCs, surface ASGR1-negative cells, and surface ASGR1-positive cells was quantified by intracellular flow cytometry. (B) Summary of results in A showing the mean percentage of ALB-positive cells by flow cytometry among unsorted HLCs, surface ASGR1-negative cells and surface ASGR1-positive cells ($n=4$ differentiations). Error bars represent s.e.m. * $P<0.05$, Student's t -test. (C) Heatmap summarizing qRT-PCR results, showing relative expression levels in ASGR1-positive cells compared with matched unsorted HLCs.

Next, we analyzed the expression of several hepatocyte-specific genes in unsorted HLCs and FACS-isolated ASGR1-positive cells from multiple differentiations of three representative hPSC lines. The average differentiation efficiency in these experiments ranged from 4.2% to 26% as measured by the percentage of ASGR1-positive cells (Fig. 2C, Fig. S2C). Particular hPSC lines generally differentiated well (e.g. FHS-1 hiPSC), whereas others differentiated with a lower efficiency (e.g. 1016 hiPSC), as expected based on prior studies of HLC differentiation propensity (Takayama et al., 2014). Hepatocyte marker genes, representing a range of *in vivo* expression patterns, from fetal expression (*AFP*) to adult liver expression [apolipoprotein A-I (*APOA1*)], were analyzed by qPCR (Su et al., 2004). We found that expression of hepatocyte marker genes was significantly higher in ASGR1-positive cells than in matched unsorted HLCs. This result was particularly pronounced for inefficient differentiations.

Global transcriptional profiling of ASGR1-positive cells

We next sought to confirm the identity of ASGR1-positive cells by global gene expression profiling. We analyzed the transcriptional profiles of matched samples of ASGR1-positive cells and unsorted HLCs differentiated from an hESC line (HUES9) and an hiPSC line (1016), as well as PHHs and HepG2 hepatoma cells (Knowles et al., 1980). Based on unbiased hierarchical clustering of all expressed genes, the global expression profiles of ASGR1-positive cells were distinct from those of matched unsorted HLCs (Fig. 3A). There was greater correlation between the expression profiles of ASGR1-positive cells from different hPSC lines ($r^2=0.959$) than there was between unsorted HLCs and ASGR1-positive cells from the same cell line ($r^2=0.944$) ($P<0.05$, Fig. S3A,B).

To further examine the effects of ASGR1 sorting, we performed differential expression analysis of the microarray data comparing ASGR1-positive cells with unsorted HLCs. We performed hierarchical clustering and heatmap visualization of all genes (probe sets) differentially expressed at a false discovery rate (FDR) of 5% (Fig. S3C) as well as of the most highly differentially expressed genes (>2 -fold difference in expression between ASGR1-positive cells and HLCs, Fig. S2C). From this analysis we observed that ASGR1-positive cells cluster more closely to PHHs than to unsorted HLCs, whereas unsorted HLCs cluster more closely to HepG2 cells than PHHs.

A paired-sample design was used for differential expression analysis to characterize the gene expression profile of ASGR1-positive cells. This analysis identified genes differentially expressed in ASGR1-positive cells relative to matched HLCs differentiated from two different hPSC lines. 766 genes were differentially expressed more than 2-fold between ASGR1-positive cells and HLCs. Of these, 330 genes were significantly more highly expressed in ASGR1-positive cells versus unsorted HLCs (Fig. S3C). Functional enrichment analysis of the genes more highly expressed in ASGR1-positive cells was performed using the PANTHER classification system (Mi et al., 2013). Statistical overrepresentation analysis revealed overrepresentation of a number of hepatocyte-related gene ontology (GO) biological processes in ASGR1-positive cells versus unsorted HLCs (Fig. S3D). Many of the overrepresented processes related to key metabolic functions performed by the liver.

Next, we assembled a panel of hepatocyte genes representing important categories of hepatic function: synthetic function (including the production of coagulation factors), energy

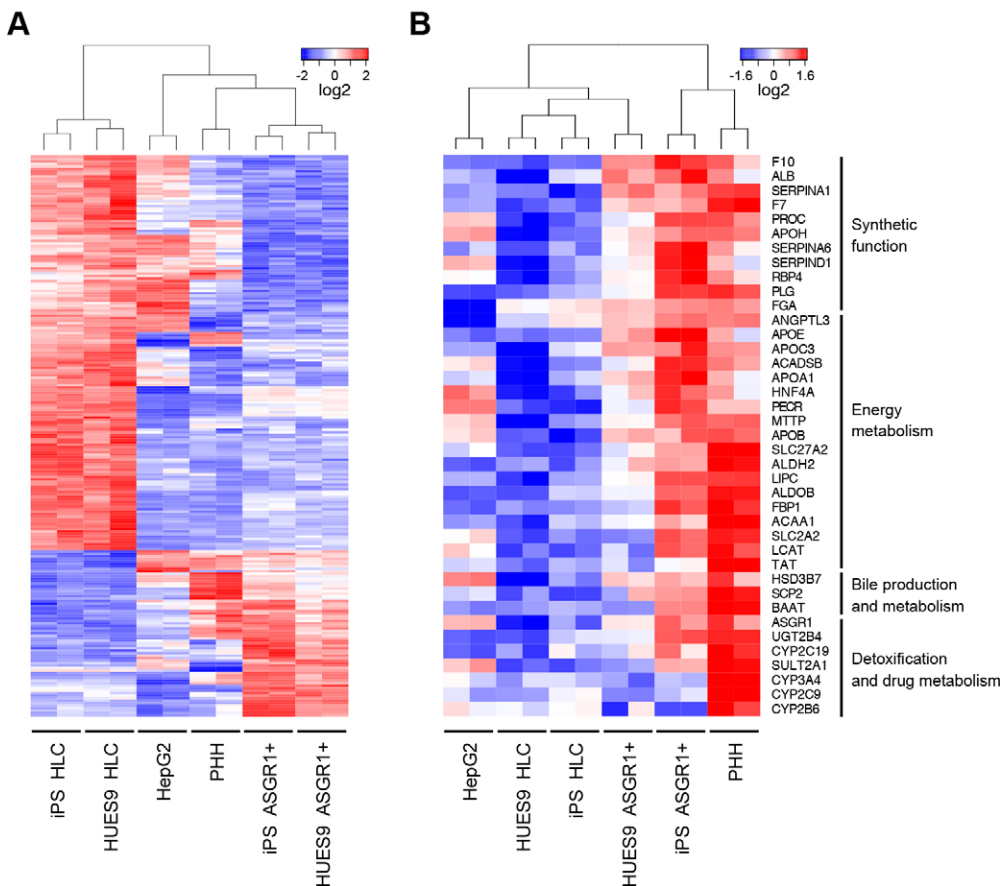


Fig. 3. Global transcriptional analysis shows that ASGR1-positive cells share a distinct transcriptional profile and are more similar than unsorted HLCs to adult hepatocytes.

(A) ASGR1-positive cells and HLCs differentiated from two different hPSC lines were compared with HepG2 hepatoma cells and adult primary human hepatocytes (PHHs) using microarrays. Shown is a heatmap of hierarchical clustering performed on genes differentially expressed more than 2-fold between ASGR1-positive cells and HLCs at a 5% FDR. 226 probe sets were differentially expressed greater than 2-fold between ASGR1-positive and unsorted HLCs at 5% FDR; 67 upregulated and 159 downregulated in ASGR1-positive versus unsorted HLCs. (B) Heatmap and hierarchical clustering of a panel of genes related to characteristic hepatic functions across the same samples as in A. Blue, below-average expression, red, above-average expression.

metabolism (including lipid and carbohydrate metabolism and lipoprotein processing), bile production and metabolism, and detoxification and drug metabolism (including metabolism of xenobiotics). The majority of these genes were expressed more highly in ASGR1-positive cells than in unsorted HLCs (Fig. 3B). Hierarchical clustering based on the expression of these hepatic functional genes suggested that ASGR1-positive cells are more similar than HLCs to PHHs (Fig. 3B). It should be noted that this trend is particularly pronounced for genes related to energy metabolism and systemic function in comparison with genes related to detoxification. Finally, we further verified that ASGR1-positive cells are more similar than unsorted HLCs to hepatocytes using gene set enrichment analysis (GSEA) (Subramanian et al., 2005). We found that 346 of 437 liver-enriched genes (79.2%) were more highly expressed in ASGR1-positive cells (Fig. S3E). We have detailed the subsets of liver-enriched genes that are both upregulated and downregulated in ASGR1-positive sorted HLCs versus unsorted HLCs in Table S1.

Replating ASGR1-enriched HLCs

To facilitate functional studies of ASGR1-positive HLCs, we optimized replating HLCs following enrichment of ASGR1-positive cells by magnetic-activated cell sorting (MACS). ASGR1 MACS-enriched HLCs adhered to standard collagen-coated 24-well plates. We examined the expression of lineage and functional hepatocyte markers in replated HLCs differentiated from hPSCs and hiPSCs and found that replated cells were positive for these markers as determined by immunofluorescence (Fig. 4A) and qPCR (Fig. S4A). This result was replicated with several hPSC lines. Finally, we assessed representative hepatocyte cellular functions in

replated HLCs in comparison to unsorted HLCs and PHHs. ALB secretion, urea secretion and CYP3A4 activity (representing secretory and detoxification functions of the liver) were significantly increased in replated HLCs versus standard unsorted HLCs (Fig. 4B). As anticipated based on prior characterization of hPSC-derived HLCs, these activities were substantially higher in PHHs. As with the dedifferentiation of primary hepatocytes in cell culture (Miyazaki et al., 1981; Rowe et al., 2010), replated ASGR1-positive HLCs dedifferentiate after 96 h and at this point no longer express the marker genes *AFP*, *FGA*, *AAT*, *ALB*, *TF* or *APOA1* (Fig. S4A) and secrete diminished quantities of ALB (Fig. S4B).

Prospective isolation of hepatocytes from heterogeneous HLC cultures based on ASGR1 surface expression is a viable solution to the problem of variable and incomplete HLC differentiation. We have shown for the first time that ASGR1, an established liver-specific protein, is expressed by a subset of ALB-positive hepatocytes upon HLC differentiation. ASGR1 FACS can be used to isolate a population of cells that express hepatocyte functional markers (*ALB* and *AAT*) even when the overall differentiation efficiency is low. ASGR1-positive sorting addresses both the impurity of HLC differentiation cultures as well as inter-cell line variability in differentiation efficiency, both of which present limitations to performing *in vitro* genetic studies using HLCs. ASGR1 localization at the plasma membrane allows sorting without fixation, making this protocol ideal for the isolation of nucleic acid and protein from purified cells. MACS and replating of ASGR1-positive HLCs eliminates the variability commonly encountered during functional studies of differentiated HLCs. This strategy ensures uniformity of cellular activities in comparison to unpurified

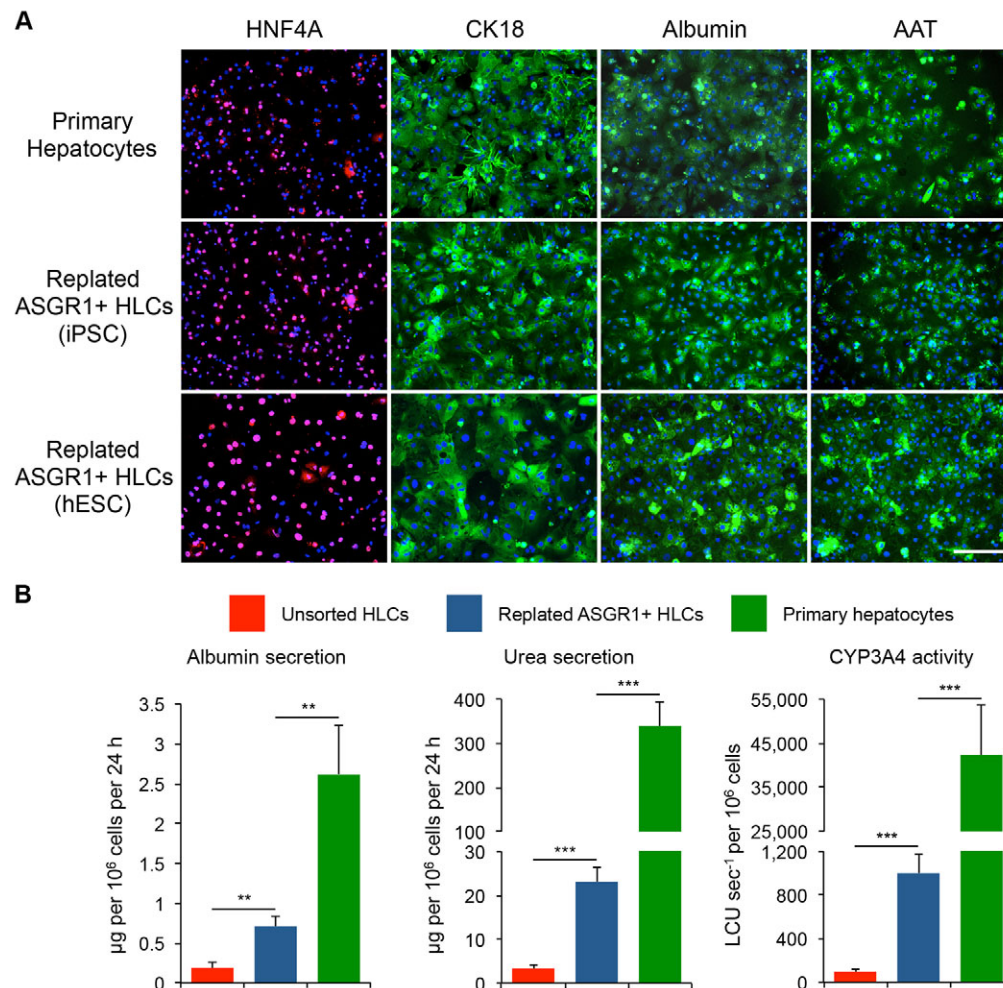


Fig. 4. HLCs can be replated following ASGR1 MACS enrichment and retain hepatocyte characteristics.

(A) Immunocytochemical staining for hepatocyte markers in PHHs and replated ASGR1 MACS-enriched HLCs. CK18, cytokeratin 18 (keratin 18, type I). Staining was performed 72 h after replating. Scale bar: 100 μm . (B) Representative assays of hepatocyte cellular functions. Assays were performed at the following time points: unsorted HLCs, day 28 of differentiation; replated HLCs, 72 h after replating on day 25 of differentiation; PHHs, 48 h after plating. $n=4$ per cell line for HLCs and $n=6$ per cell line for replated HLCs. Cryopreserved PHHs were thawed and plated ($n=4$). Error bars represent s.e.m. $**P<0.01$, $***P<0.001$, Student's *t*-test.

cells. In conclusion, purification of ASGR1-positive cells could be generally applied to isolate HLCs differentiated from hPSCs.

MATERIALS AND METHODS

Cell culture

Two hESC lines (HUES1 and HUES9; Cowan et al., 2004) and two hiPSC lines (1016 and FHS-1) were used. HUES1 and HUES9 cells are part of the NIH hESC registry; 1016 and FHS-1 were derived by the Harvard University Induced Pluripotent Stem Cell Core Facility using retroviral and Sendai viral reprogramming, respectively. Cryopreserved PHHs (HMCPS lot no. Hu8138) were cultured according to the manufacturer's instructions (Life Technologies). All cell lines were routinely tested for mycoplasma contamination. All ESC lines were maintained in accordance with ESCRO guidelines and iPSC lines were reprogrammed with full consent of donors.

Differentiation of hESCs and iPSCs into HLCs

Media

Basal differentiation medium (BDM): RPMI-1640 (Corning) plus B27 supplement plus penicillin/streptomycin (100 U/ml penicillin, 100 $\mu\text{g/ml}$ streptomycin, final concentration) (Thermo Scientific). Definitive endoderm (DE) medium: BDM with 100 ng/ml activin A and 3 μM CHIR99021. Hepatic endoderm (HE) medium: BDM with 5 ng/ml basic fibroblast growth factor (bFGF, also known as FGF2), 20 ng/ml bone morphogenetic protein 4 (BMP4) and 0.5% DMSO. IMH medium: BDM with 20 ng/ml hepatocyte growth factor (HGF) and 0.5% DMSO. Mature hepatocyte (MH) medium: hepatocyte basal medium (HBM) (Lonza) with SingleQuots added (Lonza), as well as 20 ng/ml HGF, 20 ng/ml oncostatin M, 100 nM dexamethasone and 0.5% DMSO.

Plating and differentiation

Day 1: cultures of ESCs/iPSCs were split and plated at a density of 3×10^4 cells/ cm^2 in mTESR (Stem Cell Technologies) with 4 μM ROCK inhibitor Y27632. Plating density may have to be optimized for each cell line. Days 2–4: cells were treated with DE medium. Days 5–9: cells were treated with HE medium for 5 days. Days 10–14: cells were treated with IMH for 5 days. Days 15–27: cells were treated with MH medium for 10–12 days. The medium was changed daily throughout differentiation. All cell sorting and staining experiments for the HLC stage were performed at day 25 of differentiation, whereas experiments studying the IMH stage were performed at day 14 of differentiation.

Immunocytochemistry

The following primary and secondary antibodies were used for immunocytochemical staining: AAT (MA1-90438, Thermo Scientific), ALB (A80-129A, Bethyl), ASGR1 (clone 8D7, BD Biosciences), CK18 (ab82254, Abcam) and HNF4 α (ab92378, Abcam) primary antibodies at 1:250, and donkey anti-goat IgG Alexa Fluor 555 (A-21432, Thermo Scientific), donkey anti-mouse IgG Alexa Fluor 488 (A-21202, Thermo Scientific) and donkey anti-rabbit IgG Alexa Fluor 488 (A-21206, Thermo Scientific) secondary antibodies (all Life Technologies) at 1:1000. Hoechst (1:5000; Life Technologies) was used for nuclear staining. Staining in unsorted HLCs was visualized using an LSM 700 confocal microscope (Carl Zeiss) and an inverted Eclipse Ti microscope (Nikon).

Intracellular flow cytometry

For flow cytometry analysis, differentiated cells were fixed and stained using the Cytofix/Cytoperm Kit (BD) following the manufacturer's instructions.

The primary and secondary antibodies described above were used at experimentally optimized dilutions. Additional antibodies used in flow cytometry experiments were mouse IgG1 isotype control (554121, BD; 1:20), donkey anti-mouse IgG Alexa Fluor 594 (A-21203, Thermo Scientific; 1:500), donkey anti-goat IgG Alexa Fluor 488 (ab150129, Abcam; 1:500) and donkey anti-goat IgG Alexa Fluor 647 (A-21447, Thermo Scientific; 1:500). Cells were analyzed using an LSR II cytometer (BD) and FlowJo software.

FACS of HLCs

After day 12 of feeding with MH medium, HLCs were washed with Dulbecco's phosphate-buffered saline (DPBS) and treated with 0.25% trypsin-EDTA. Cells were treated with a Stempro EZ Passage passaging tool (Life Technologies) and incubated at 37°C for 15 min. After dissociation, remaining cells were gently scraped from the dish and filtered through a 100 µm mesh. Cells were stained with a PE-conjugated ASGR1 antibody (8D7, BD, 563655) or a PE-conjugated mouse IgG1κ isotype control antibody (BD, 551436) according to manufacturer's dilution instructions. After incubation, cells were washed twice with PBS. Alternatively, in some experiments cells were stained with unconjugated ASGR1 primary antibody or mouse IgG1 isotype control, washed, and stained with donkey anti-mouse IgG Alexa Fluor 488 secondary antibody (as above). ASGR1-positive cells were purified by FACS using a FACSaria II (BD).

RNA isolation and qRT-PCR

RNA was isolated using Trizol (Life Technologies). qPCR was performed using TaqMan Gene Expression Master Mix and TaqMan gene expression assays (Life Technologies) for the following: *RPLP0* (reference control gene), *AFP*, *CYP3A7*, *FGA*, *ALB*, *AAT*, *TF* and *APOA1*.

Microarray gene expression profiling

U219 gene expression arrays (Affymetrix) were used according to the manufacturer's instructions. These data have been uploaded to Gene Expression Omnibus (GEO) under accession number GSE77086.

Preprocessing and hierarchical clustering

Microarrays were normalized and background corrected using the robust multi-array (RMA) method in the Bioconductor affy package in R v3.1.2 (Gautier et al., 2004). Normalized array values were reported on a log₂ scale and probe sets with low expression values across all samples (log₂ intensity <2.5) were filtered out. Unsupervised hierarchical clustering was performed using the *hclust* function in R. The statistical significance of clustering results was estimated by multiscale bootstrap resampling using the *pvcust* function with 10,000 iterations.

Standard differential expression analysis and heatmap generation

Analysis was performed using Transcriptome Analysis Console software (Affymetrix). FDR-adjusted *P*-values were calculated based on the Benjamini-Hochberg method and 5% FDR cutoff was used. Heatmaps were created using the *heatmap.2* function in R, with accompanying dendrograms drawn based on Euclidean distance.

Paired-sample differential expression analysis, functional enrichment testing of differentially expressed genes and GSEA

The R/Bioconductor limma package was used to fit linear models for each gene, utilizing a paired-sample design. Moderated *t*-statistics, log fold change and *P*-values were calculated. To correct for multiple hypothesis testing, FDR-adjusted *P*-values were calculated by the Benjamini-Hochberg method. Probe sets were filtered by FDR<5% and fold change >2 (1079 probe sets). Functional enrichment analysis was performed using the PANTHER resource. GSEA (Subramanian et al., 2005) was performed using GSEA software (Broad Institute, Cambridge, MA, USA) and a list of liver-enriched genes (Yang et al., 2011).

Analysis of published gene expression data

Published microarray gene expression data were obtained from BioGPS.org and the GEO database: accession numbers GSE25417 (DeLaForest et al., 2011) and GSE1133 (Su et al., 2004). Analysis and heatmap generation were conducted using GENE-E software (Broad Institute).

ASGR1 MACS and replating of HLCs

Cells were released as described for flow cytometry analysis. After blocking, anti-ASGR1 antibody (8D7, BD; 1:150) was added. After incubation on ice for 30 min, cells were pelleted (5 min, 233 *g*) and resuspended in cold MACS buffer with anti-mouse IgG microbeads (1:20; Miltenyi Biotec). After incubation on ice for 30 min, cells were washed and then passed through a 100 µm filter. Cells were then sorted using an autoMACS Pro separator (Miltenyi Biotec). After sorting, cells were pelleted and resuspended in HLC replating medium: hepatocyte basal medium with SingleQuots supplements, HGF (20 ng/ml), dexamethasone (100 nM), ROCK inhibitor (4 µM), penicillin/streptomycin and gentamicin (50 µg/ml). Cells were plated on collagen-coated 24-well culture dishes (A1142802, Thermo Scientific) by centrifugation of the plate at 100 RCF for 15 min at 37°C. Cells were replated at 15,000 cells per cm² and thereafter maintained in HLC replating medium without ROCK inhibitor. Cells were used in functional assays no later than 72 h after replating, owing to cell dedifferentiation during prolonged culture.

Cellular assays of hepatocyte functions

Cellular assay results were normalized to the number of live cells as determined by hemocytometer cell counting with Trypan Blue staining. ALB secretion was quantified using an enzyme-linked immunosorbent assay (Bethyl Laboratories, E80-129) according to the manufacturer's instructions. Urea production was quantified using the QuantiChrom Urea Assay Kit (BioAssay Systems) following the manufacturer's instructions. Cytochrome activity was quantified using the P450-Glo CYP3A4 assay (Luc-PFBE) (Promega) following the manufacturer's instructions.

Statistical analysis

Statistical significance was assessed using standard Student's *t*-test (two-tail); *P*<0.05 was considered statistically significant. Experiments were performed in triplicate unless otherwise noted.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

D.T.P. and C.R.W. designed and performed experiments and wrote the manuscript. C.A.H. and M.F. designed and performed experiments. F.X. performed experiments. C.E.B. reprogrammed iPSC lines. K.M. supported D.T.P. C.A.C. designed experiments and guided the study.

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Data availability

Microarray data are available at Gene Expression Omnibus under accession number GSE77086.

Supplementary information

Supplementary information available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.132209/-/DC1>

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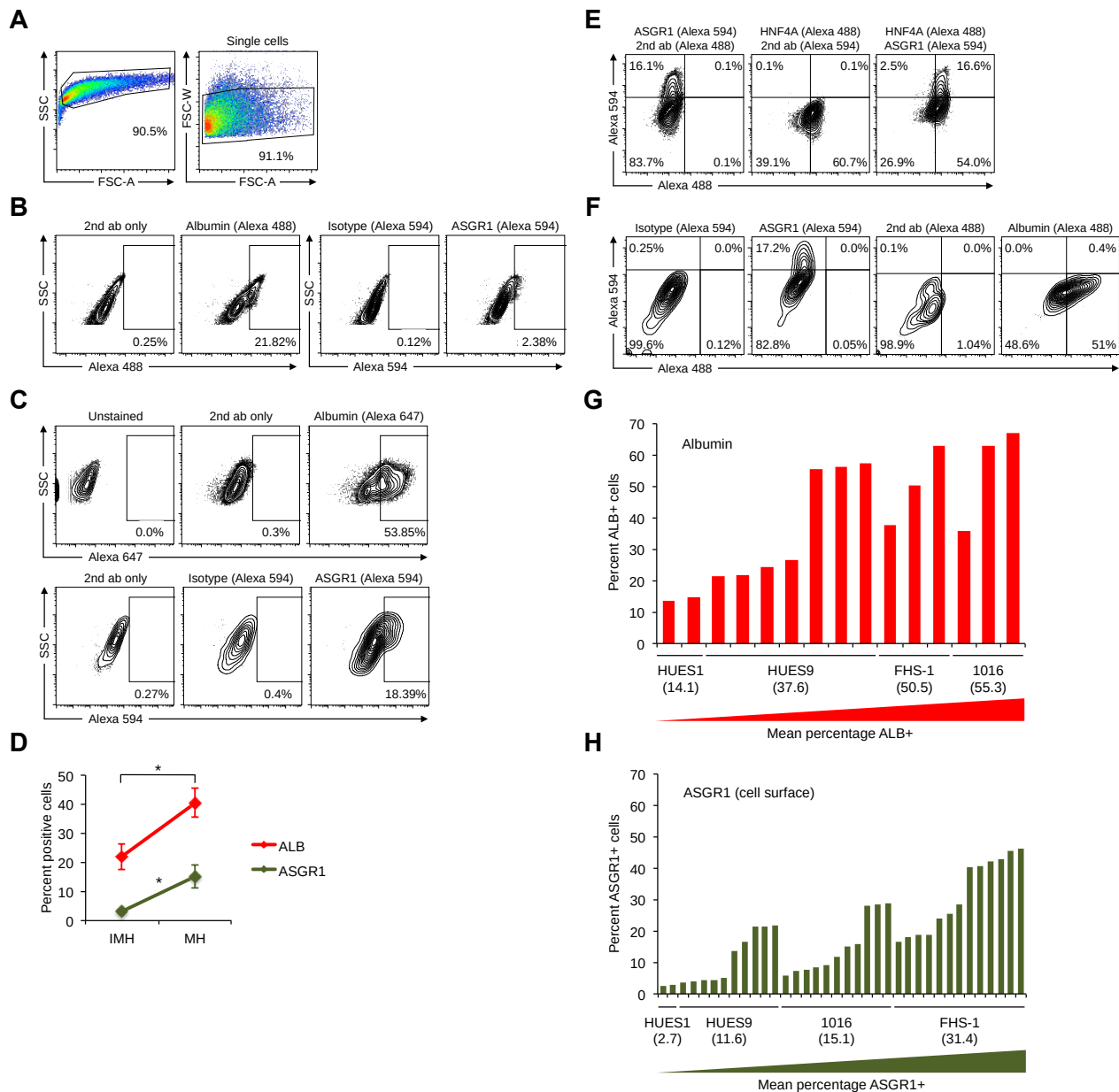


Figure S1. Flow cytometry analysis of HLC differentiation, related to Figure 1. (A) Gating used for flow cytometry analyses; single cells were defined by FSC-A/SSC (cell size and granularity) and FSC-W (cell width) to exclude debris, cell clumps, and doublets. (B) Albumin and ASGR1 expression after the IMH differentiation stage with corresponding staining controls. Alexa 488 and Alexa 594 conjugated secondary antibodies were used with albumin and ASGR1 primary antibodies respectively; gating for albumin and ASGR1 positive cells was performed using secondary-only and isotype-control staining conditions

respectively. (C) Albumin and ASGR1 expression after the MH differentiation stage with corresponding staining controls. Alexa 647 and Alexa 594 conjugated secondary antibodies were used with albumin and ASGR1 primary antibodies respectively; gating for albumin and ASGR1 positive cells was performed using secondary-only and isotype-control staining conditions respectively. A secondary-only staining control is also shown for ASGR1. (D) Kinetics of albumin and ASGR1 expression during the final stages of HLC differentiation as determined by intracellular flow cytometry. Shown are mean percent positive cells at the IMH and MH stages based on multiple independent differentiations ($n = 5 - 15$ replicates per marker, per stage). Error bars represent s.e.m. Asterisks indicate statistically significant differences in mean percentage of positive cells at the IMH and MH stages for each marker by Student's t -test. *, $P < 0.05$. (E) Flow cytometry analysis of ASGR1 and HNF4A co-expression with staining controls. Alexa 594 and Alexa 488 conjugated secondary antibodies were used for ASGR1 and HNF4A staining respectively. Gating was performed based on secondary-only (fluorescence minus one) staining conditions. (F) Flow cytometry analysis of ASGR1 and albumin co-expression with staining controls. Alexa 594 and Alexa 488 conjugated secondary antibodies were used for ASGR1 and albumin staining respectively. Gating for ASGR1 and albumin positive cells was performed based on isotype-control and secondary-only staining conditions respectively. (G-H) Efficiency of HLC differentiation with four different hPSC lines over multiple independent experiments. Shown are percent albumin-positive (G) and surface ASGR1-positive (H) cells determined by flow cytometry analysis after the MH differentiation stage, illustrating a range of differentiation efficiencies.

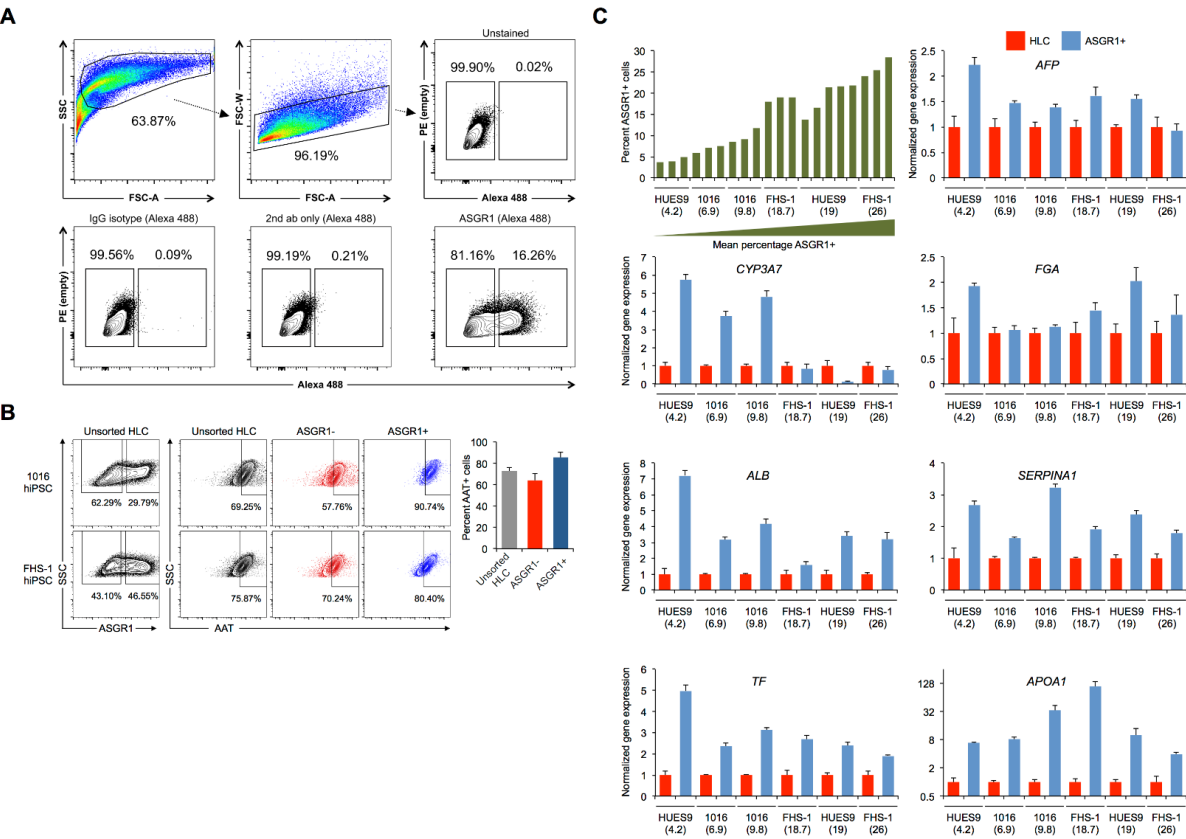


Figure S2. Detailed results of ASGR1 FACS and hepatocyte marker gene expression analysis, related to Figure 2. (A) Strategy and staining controls for FACS isolation of ASGR1+ HLCs. Shown are results of a representative differentiation and FACS experiment. (B) Left: two different hPSC lines were differentiated to HLCs. The percentage of cells expressing the hepatocyte marker alpha-1 antitrypsin (AAT) among unsorted HLCs, surface ASGR1-negative cells, and surface ASGR1-positive cells was quantified by intracellular flow cytometry. Right: mean percent AAT-positive cells by flow cytometry, among unsorted HLCs, surface ASGR1-negative cells, and surface ASGR1-positive cells ($n = 2$ differentiations). Error bars represent s.e.m. (C) ASGR1 FACS and hepatocyte marker gene expression analysis by qRT-PCR. Gene expression data is displayed as a heatmap in Figure 2C. Three different hPSC lines were differentiated to HLCs, with two independent differentiations performed per cell line (differentiations performed in triplicate or greater, $n = 6 - 8$ biological replicates per cell line). Data are arranged according to mean percent ASGR1+ cells obtained in each differentiation. Green bars: percentage surface ASGR1+ cells after the MH

differentiation stage. All other graphs show qRT-PCR gene expression analysis in unsorted HLCs (“HLC,” red bars) and ASGR1+ cells (“ASGR1+,” blue bars) isolated by FACS. Expression levels are relative to *RPLP0* expression; gene expression levels in ASGR1+ cells were normalized to level in unsorted HLCs. Shown are normalized mean expression levels for each differentiation ($n = 3 - 5$ paired biological replicates per differentiation). Error bars represent s.e.m.

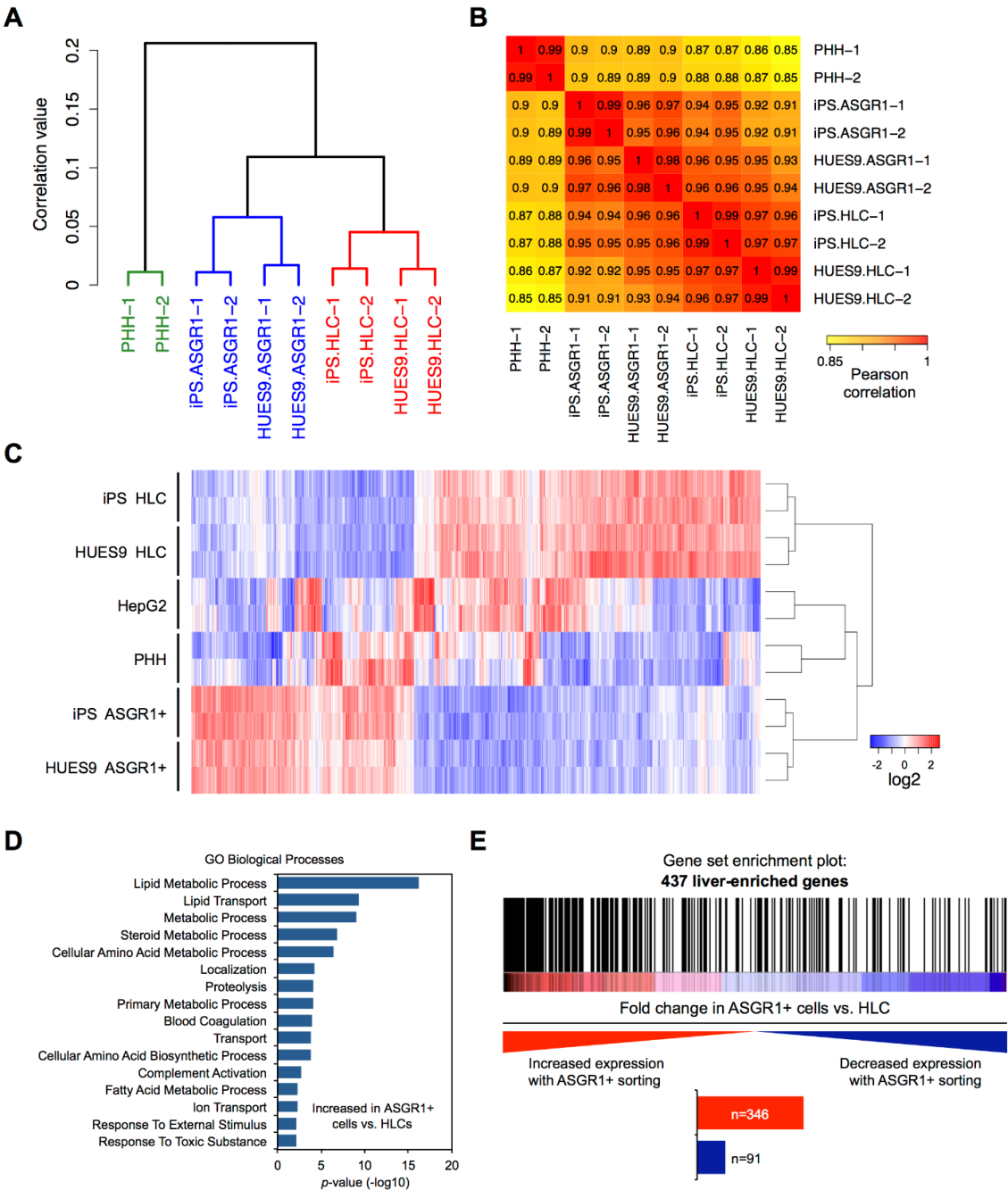


Figure S3. Additional analyses of microarray gene expression profiling data from ASGR1-positive cells, unsorted HLCs, primary human hepatocytes, and HepG2 hepatoma cells, related to figure 3. (A) Hierarchical clustering of ASGR1-positive, HLC, and PHH samples based on all genes measured by transcriptional microarray and expressed above background. (B) Heatmap showing pairwise Pearson correlation values for ASGR1-

positive, HLC, and PHH samples based on the same expression data used in part A. Yellow, orange, and red color denotes lower, intermediate, and higher correlation respectively. (C) Heatmap of hierarchical clustering performed on all genes differentially expressed between ASGR1-positive cells and HLCs at a 5% FDR. Blue, below average expression, red, above average expression. 813 probesets differentially expressed between ASGR1+ and unsorted HLC at 5% FDR; 318 upregulated and 495 downregulated in ASGR1+ vs. unsorted HLCs respectively. (D) Functional enrichment analysis of genes differentially expressed in ASGR1-positive cells relative to HLCs. (E) Gene set enrichment analysis (GSEA). Microarray gene expression data was arranged based on greater average expression in ASGR1-positive cells (red color) or unsorted HLCs (blue color). Vertical black bars represent genes within the liver-enriched gene set.

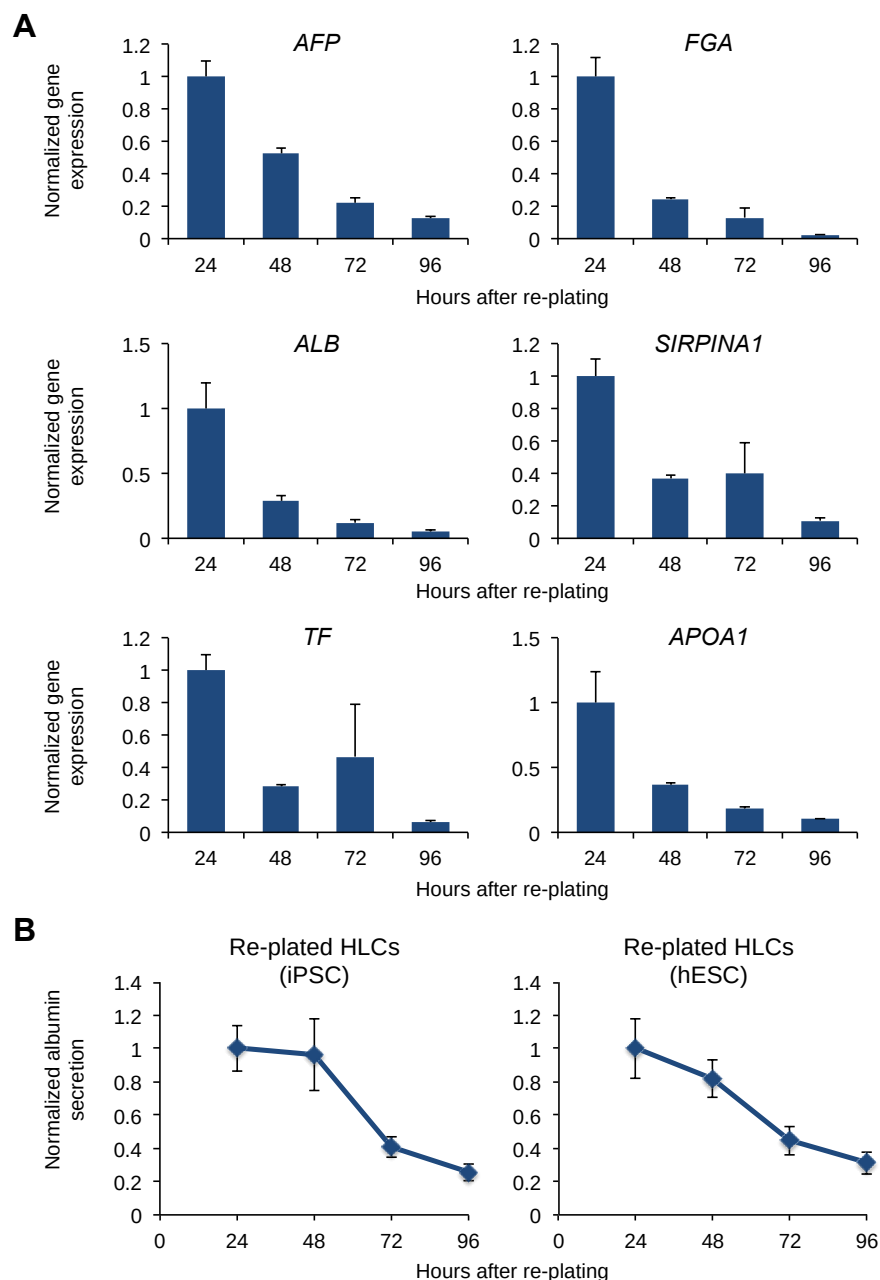


Figure S4. Hepatocyte marker gene expression and albumin secretion declines as anticipated following re-plating of ASGR1 MACS-enriched HLCs. (A) Changes in hepatocyte marker gene expression over time were determined by qRT-PCR after re-plating HLCs differentiated from a representative hESC line. Gene expression levels were calculated relative to *RPLP0* expression and normalized to gene expression level at 24 hours post re-plating for each gene ($n = 3$ differentiation wells per time point). Error bars represent s.e.m. (B)

Quantification of albumin secretion by ELISA following re-plating of HLCs differentiated from two hPSC lines. Albumin concentration at the indicated time points were calculated using a standard curve and normalized to the level at 24 hours post re-plating for each cell line ($n = 3$ differentiation wells per cell line, per time point). Medium was collected after 24 hours in culture for each time point. Error bars represent s.e.m.

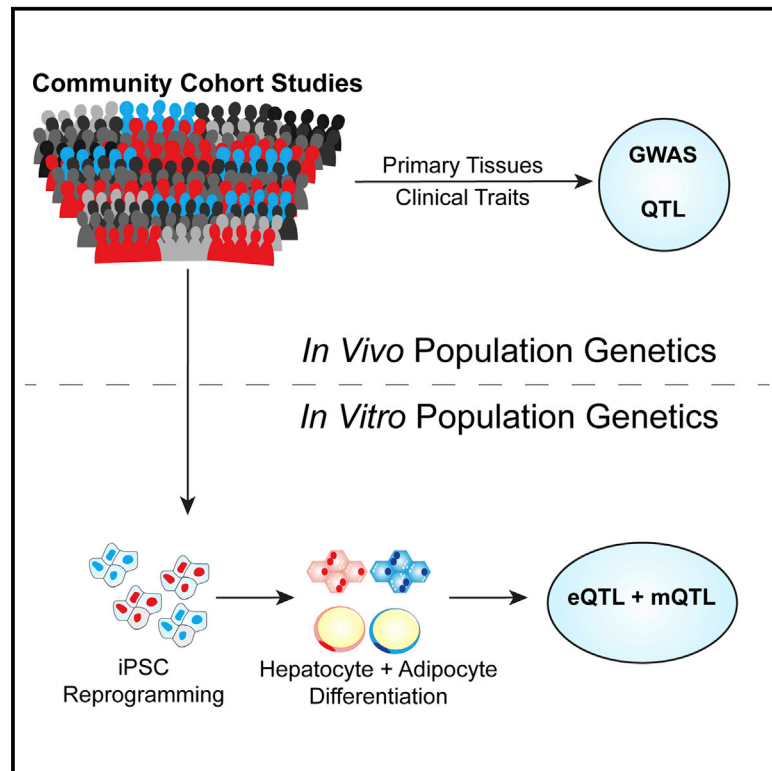
Table S1. Distribution of liver-enriched genes in gene set enrichment analysis (GSEA) comparing ASGR1+ cells and unsorted HLCs, related to Figure S3E.

Subset of liver-enriched genes with greater expression in ASGR1+ cells. 205 genes contributed most strongly to the enrichment result (core enrichment). 346 of 437 genes had greater expression in ASGR1+ cells vs. unsorted HLCs.						Liver-enriched genes with lower expression in ASGR1+ cells. 91 of 437 genes had lower expression in ASGR1+ cells vs. unsorted HLCs		
AADAC	ASGR1	FBP1	LEPR	SEPHS2	TTR	ABCB4	FMO4	SEC14L2
ABCG8	ASGR2	FETUB	LIPC	SERPINA10	UGT2B4	ABCG5	GCGR	SEC14L4
ACAA1	ASS1	FGA	MAT1A	SERPINA4	UGT3A1	ACAT2	GHR	SLCO1B1
ACAA2	ATF5	FGB	METTL7B	SERPINA6	VTN	ACOT12	GLS2	SOD1
ACADSB	AZGP1	FGFR4	MTHFS	SERPINA7	ZNHIT1	ANXA10	GLT1D1	SPP2
ACAT1	BAAT	FGG	MTTP	SERPINC1		APOC4	GNE	STEAP3
ACMSD	BPHL	FGL1	MUT	SERPIND1		AQP9	GNPNAT1	THPO
ACOX1	BRP44	FMO5	NAT8	SERPINF2		AS3MT	GPR126	TNFSF14
ACOX2	C1S	G6PC	NEK6	SERPING1		ATF7IP2	GRHPR	TUBB1
ACSL1	C5	GATM	NIPSNAP1	SHMT1		BCO2	GSTZ1	UROCI
ACY1	C6	GC	NIT2	SHMT2		C4BPA	GPS2	ZNF281
ADH1A	CD302	GCHFR	OIT3	SLC10A1		C4BPB	HAGH	
ADH6	CDO1	GGH	ORM1	SLC13A5		CCL16	HAMP	
ADK	CFH	GSTO1	OTC	SLC22A7		CD5L	HLF	
AFF4	CFI	HABP2	PAH	SLC22A9		CDC37L1	HPS3	
AFM	CIDEB	HMGCL	PCBD1	SLC25A13		CES1	HSD11B1	
AGTR1	CLDN1	HMGCS2	PCCB	SLC27A2		CES2	IBTK	
AGXT	CPB2	HNF4A	PCK2	SLC2A2		CFP	IGFALS	
AGXT2	CPN1	HP	PEBP1	SLC30A1		CLEC4M	ITIH4	
AHSG	CPN2	HPD	PECR	SLC35D1		COLEC11	KMO	
AIG1	CPS1	HPN	PEMT	SLC38A3		CP	LARP4	
AKR1A1	CREB3L3	HPX	PGRMC1	SLC38A4		CRP	LECT2	
AKR1D1	CRYL1	HRSP12	PHYH	SLC39A14		CTH	LPIN2	
ALAS1	CYB5A	HSD17B4	PIPOX	SLC41A2		CYP1A2	MAMDC4	
ALB	CYP3A7	HSD3B7	PKLR	SLC43A1		CYP26A1	MASP1	
ALDH6A1	CYP4A11	HSPE1	PLA2G12B	SLCO2B1		CYP2A6	MASP2	
ALDH8A1	CYP4V2	ID2	PLG	SORD		CYP2B7P1	MBL2	
ALDOB	CYP8B1	IGFBP1	PNPLA3	SPRYD4		CYP2C8	MCL1	
AMBP	DCXR	IL1RAP	PRAP1	ST6GAL1		CYP2D6	MTHFD1	
ANG	DDT	INHBE	PRDX4	SULT2A1		CYP2E1	MYO1B	
ANGPTL3	DECR1	INSIG1	PROC	TAT		CYP39A1	N4BP2L1	
APOA1	EPHX1	ITIH1	PROS1	TDO2		CYP4A22	PHLDA1	
APOA2	ETFB	ITIH2	PROX1	TFR2		DEFB123	PLGLB2	
APOB	F10	KHK	PROZ	TM4SF4		DEPDC7	PON1	
APOC2	F13B	KLB	PXMP2	TM4SF5		DHRS1	PON3	
APOC3	F2	KLKB1	RBP4	TMEM176A		DMGDH	PPAPDC2	
APOE	F5	KNG1	RCL1	TMEM176B		ERRFI1	PZP	
APOH	F7	LASS2	SAA4	TMEM56		ETFDH	RTP3	
APOM	F9	LBP	SC5DL	TMPRSS6		FAM167B	SCUBE3	
ARG1	FAM96A	LCAT	SCP2	TP53INP1		FMO3	SDS	

Cell Stem Cell

Induced Pluripotent Stem Cell Differentiation Enables Functional Validation of GWAS Variants in Metabolic Disease

Graphical Abstract



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In Brief

As part of the NHLBI NextGen consortium, Warren et al. show that analyzing the cellular pathophysiology of cells differentiated from multiple lines of an iPSC library is a promising complementary approach in the functional analysis of GWAS variants.

Highlights

- Peripheral blood cells from study participants were reprogrammed to iPSCs
- iPSC lines differentiated into white adipocytes and hepatocyte-like cells
- Transcriptomic and metabolomic profiling highlights cellular phenotypes
- Differentiated cells reproduce expression of variation-associated loci



Induced Pluripotent Stem Cell Differentiation Enables Functional Validation of GWAS Variants in Metabolic Disease

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SUMMARY

Genome-wide association studies (GWAS) have highlighted a large number of genetic variants with potential disease association, but functional analysis remains a challenge. Here we describe an approach to functionally validate identified variants through differentiation of induced pluripotent stem cells (iPSCs) to study cellular pathophysiology. We collected peripheral blood cells from Framingham Heart Study participants and reprogrammed them to iPSCs. We then differentiated 68 iPSC lines into hepatocytes and adipocytes to investigate the effect of the 1p13 rs12740374 variant on cardiometabolic disease phenotypes via transcriptomics and metabolomic signatures. We observed a clear association between rs12740374 and lipid accumulation and gene expression in differentiated hepatocytes, in particular, expression of *SORT1*, *CELSR2*, and *PSRC1*, consistent with previous analyses of this variant using other approaches. Initial investigation of additional SNPs also highlighted correlations with gene expression. These findings suggest that iPSC-based population studies hold promise as tools for the functional validation of GWAS variants.

INTRODUCTION

Genome-wide association studies (GWASs) provide insight into the effects of common genetic variants on physiology and disease. By coupling this technological breakthrough with the clinical resource of large community-based longitudinal studies, GWASs have revealed thousands of novel genetic mechanisms underlying disease and other traits (Teslovich et al., 2010; Visscher et al., 2012). In addition to associating coding mutations and novel genes with disease, GWASs have identified subtle phenotypes driven by non-coding SNPs by virtue of their large sample size and unbiased study design. Hundreds of non-coding SNPs have been implicated by GWASs in the control of blood lipid concentrations and/or myocardial infarction (MI) that results from dyslipidemia. One of the loci most significantly associated with variance in plasma concentration of low-density lipoprotein cholesterol (LDL-C) and MI is located on chromosome 1p13. An expression quantitative trait locus (eQTL) analysis of non-coding SNPs at 1p13 correlated the minor haplotype with increased gene expression specifically in human liver (Musunuru et al., 2010). One SNP, rs12740374, was responsible for the minor haplotype-associated increase in gene expression of *SORT1*, *PSRC1*, and *CELSR2*. The minor allele of rs12740374 mediates this effect by creating a CAAT-enhancer-binding protein (C/EBP) recognition site (Musunuru et al., 2010). We found that increasing hepatic *SORT1* expression resulted in decreased secretion of LDL-C, explaining the decreased risk of MI

associated with this common non-coding variant. This finding prompted us to explore whether induced pluripotent stem cells (iPSCs) could be used to model the effect of this non-coding SNP on hepatic lipid phenotypes. In addition to employing genome editing to delete the causal SNP in an iPSC line, we have also, for the first time, performed a hepatic eQTL analysis using a large cohort of iPSCs from Framingham Heart Study (FHS) participants of variable genetic backgrounds. By employing rigorous reprogramming and differentiation efficiency controls as well as cell purification strategies, we have demonstrated that iPSCs are indeed capable of recreating a hepatocyte-specific eQTL and, in so doing, have garnered insights into hepatic lipid biology driven by the common non-coding variant rs12740374.

iPSCs can be directly derived from the somatic cells of any donor prospectively identified to carry a genotype of interest (Park et al., 2008). This versatile feature makes iPSCs an ideal platform for the study of Mendelian or monogenic disease. The use of iPSCs for the study of common SNPs with subtle effects on metabolic phenotypes may require large-scale reprogramming efforts coupled with a fine control of directed differentiation techniques. As a proof of principle for future “GWAS in a dish” approaches to common disease genetics, we chose to model the eQTL previously characterized at the 1p13 locus that is associated with increased *SORT1* expression. In vivo, an increase in *SORT1* expression occurs specifically in the liver tissue but not in the adipose depots. This modeling experiment involved the reprogramming of somatic cells from 34 donor participants selected for their homozygous major or minor genotype at the 1p13 SNP rs12740374 and differentiation of the cognate iPSCs into both hepatocytes and white adipocytes. RNA sequencing, metabolomics, and integrative statistical analyses were employed to analyze the effect of the 1p13 genotype on gene expression and metabolism. This experimental approach yielded molecular insights into the protective mechanism of the 1p13 SNP, identifying dysregulation of lipid accumulation and a number of novel *trans*-eQTLs specifically in differentiated hepatocytes. These results demonstrate the capacity for iPSCs to recapitulate expression quantitative trait locus analyses in vitro and yield novel pathophysiological insights. With improved reprogramming and differentiation protocols, this strategy may be used for discovery and functional studies of common disease-related genetic variants in the future.

RESULTS

Recruitment of the Donor Cohort and Reprogramming of Peripheral Blood Mononuclear Cells

The FHS offspring cohort, consisting of offspring and spouses of offspring of the original FHS cohort, was enrolled in 1971 as a prospective study of heart disease epidemiology (Kannel et al., 1979). This cohort of 5,124 individuals of European descent has been serially examined at regular intervals (Kannel et al., 1979). The offspring database now includes a large number of biomarker measurements in parallel with subclinical and clinical phenotypes as well as genome-wide genotyping and genome sequencing data measured in many participants. We collected T cells from consenting offspring cohort participants in the ninth examination cycle (2011–2014). We reprogrammed cryopreserved peripheral blood mononuclear cells (PMBCs) from a

selected subset of 17 participants homozygous for the major haplotype at the 1p13 locus and 17 participants homozygous for the minor haplotype at the 1p13 locus (Figure 1A). The non-replicating Sendai virus (SeV) was used to introduce the reprogramming factors OCT4, KLF4, SOX2, and c-Myc into T cells (Takahashi et al., 2007). Five to seven clonal cell lines were isolated from the reprogrammed cells for each donor participant, and two were further expanded and characterized, resulting in 68 iPSC lines.

Each iPSC line was submitted to a battery of tests for quality control purposes. The expression of key pluripotency markers (OCT4, Nanog, SSEA3, SSEA4, and Tra-1-60) was assessed using immunofluorescence (Figure 1B). qRT-PCR was used to assess the expression of six key pluripotency genes (*DNMT3B*, *TERT*, *NANOG*, *OCT4*, *REX1*, and *SOX2*). The expression of each of these pluripotency genes was detected in all 68 iPSC lines and was equivalent to the expression of these pluripotency genes by an existing panel of human embryonic stem cell (hESC) lines (Figure 1C). G-band karyotyping was performed to ensure that these cell lines had not incurred chromosomal abnormalities (Figure S1A). Sixty-four of the 68 iPSC lines carried no chromosomal abnormalities (Table S1). Two cell lines carried three X chromosomes, and two cell lines carried a non-pathological inversion on chromosome 11. In both cases, these abnormalities occurred in clonal cell lines derived from the same participant, suggesting that these chromosomal abnormalities stem from the donor participant's genome rather than the reprogramming process. These chromosomal abnormalities had no effect on differentiation efficiency (Table S1) because efficiencies of these cell lines were within 1 SD of the mean. Similarly, expression of adipocyte and hepatocyte-like cell (HLC) marker genes was not affected in these cell lines. A common concern of reprogramming is the persistence of transgenic reprogramming factors after pluripotency is achieved. The delivery method we used relied on the non-integrating SeV, which is diluted out of the cellular population through passaging. We assessed the presence of SeV-delivered transgenes via qRT-PCR at varying passages and, ultimately, could not detect the persistence of SeV RNA in our cell lines (Figure S1B). All cell lines were characterized by short tandem repeat (STR) fingerprinting, and all were deemed matched to their donor of origin by this analysis (data not shown).

We employed the embryoid body (EB) Scorecard assay (Bock et al., 2011; Tsankov et al., 2015) as a measure of iPSC differentiation potential prior to differentiation into functional cell types. After differentiation into EBs and plating onto gelatin, RNA was harvested, and the expression of 92 genes was assayed by the Taqman-based Scorecard assay. These genes were chosen for their specificity in expression to the three germ layers and pluripotent stem cells. EBs from all cell lines lost expression of pluripotency genes and expressed markers for ectoderm, mesoderm, and endoderm (Figure 1D). Some variability was evident between individual iPSC lines in their capacity to differentiate into the three germ layers (Figure S1C), but every cell line expressed genes associated with each germ layer during EB differentiation, confirming their pluripotent state.

Programming of iPSCs into White Adipocytes

We differentiated white adipocytes from each of our 68 iPSC lines. To control for variability in differentiation, we performed

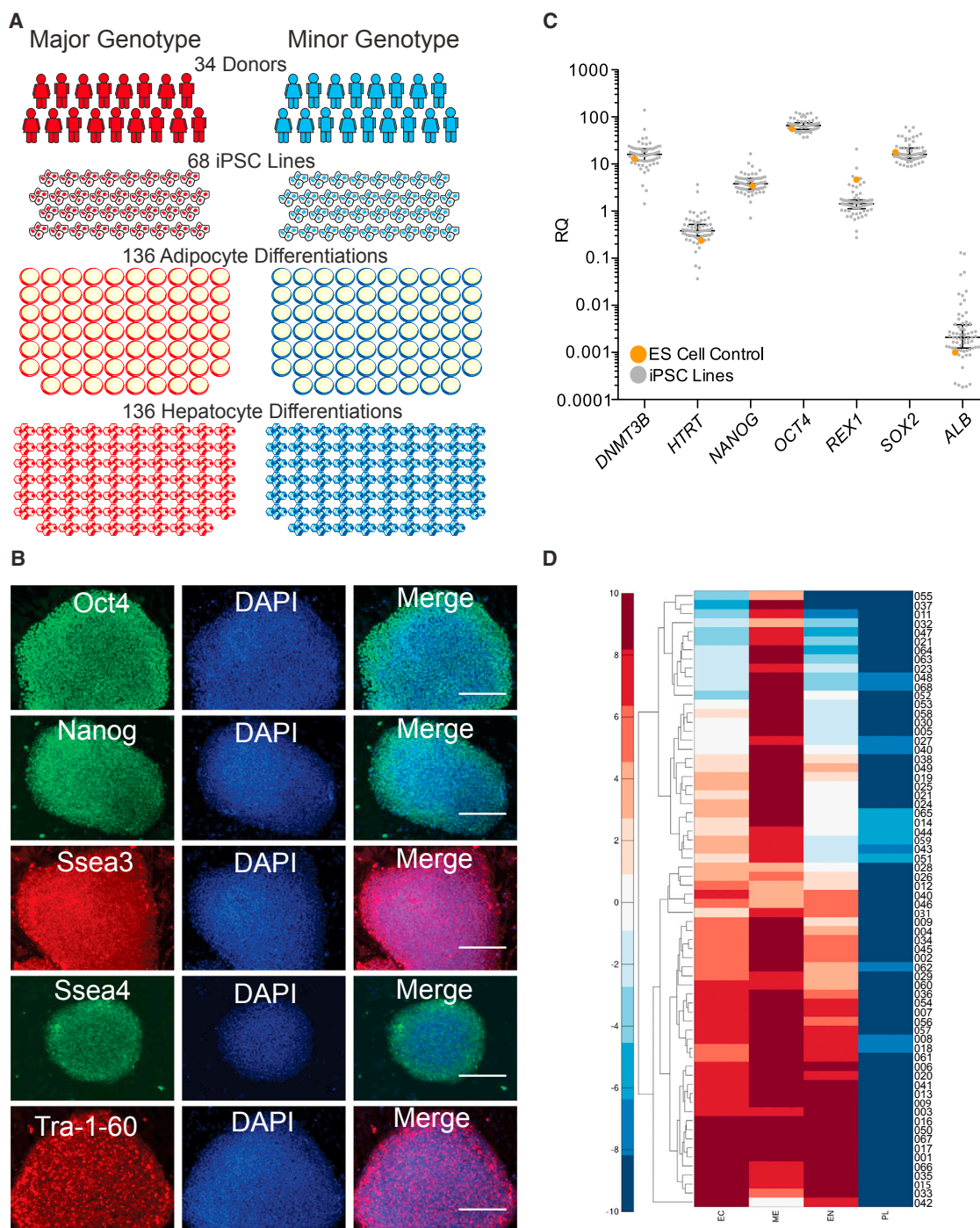


Figure 1. Research Strategy and Reprogramming of 34 Donor Participant Samples into iPSCs

(A) Blood samples from 17 donors of the major genotype and 17 donors of the minor genotype for rs12740374 were reprogrammed into two clonal iPSC lines each. These cell lines were then differentiated into both white adipocytes and HLCs for metabolic and transcriptomic profiling.

(B) Representative immunofluorescence indicating expression of pluripotency markers by iPSC colonies. Scale bars, 200 μ m.

(C) qRT-PCR was performed to demonstrate expression of pluripotency genes by each cell line (ALB served as a negative control) ($n = 68$). Orange dots, human embryonic stem cell line control value; line, mean; bars, SEM. Relative quantification (RQ) as described in the [STAR Methods](#).

(D) Hierarchical clustering of cell lines (rows) by Euclidean distance similarity of their Scorecard differentiation potential (columns). The Scorecard differentiation potential was computed as in [Tsankov et al. \(2015\)](#) using gene expression signatures of ectoderm (EC), mesoderm (ME), endoderm (EN), and pluripotent (PL) marker genes. The legend on the left displays the range of scores relative to a reference set of 23 human PSC lines. Clone IDs are listed on the right y axis.

See also [Figure S1](#).

two experimental replicates for each cell line, resulting in 136 total replicates of white adipocyte differentiation (Figure 1A). We followed a published white adipocyte programming protocol (Ahfeldt et al., 2012; Lee and Cowan, 2014), relying on differentiation into mesenchymal progenitor cells (MPCs) followed by programming into white adipocytes by a lentivirally delivered and doxycycline-inducible peroxisome proliferator activated receptor gamma-2 (PPARG2) (Figure 2A).

MPCs were produced in batches, cryopreserved, and RNA-profiled prior to white adipocyte differentiation. MPC expression of key mesoderm marker genes (*CD44*, *NT5E*, and *ENG*) was confirmed by qRT-PCR (Figure S2B). Two MPC differentiations were performed for each clonal cell line, and the MPC batch with the highest steady-state expression of these three mesoderm genes was chosen for programming with PPARG2. PPARG2 was induced by doxycycline for 16 days, and, 6 days after withdrawal of doxycycline, differentiation efficiency was assessed by immunofluorescence (Figure 2B). C/EBP-alpha works in concert with PPARG2 to promote adipogenesis and establish white adipocyte maturity, and its presence is used here as a proxy for differentiation efficiency (Freytag et al., 1994; Wu et al., 1999). A high-content automated imaging platform was used to analyze the cells in 25 fields of a representative well of differentiated white adipocytes (Figure S2A). Differentiation was quantified for each replicate (Figure 2C). The mean cumulative differentiation efficiency over all 136 differentiation experiments was 60.4% and the median differentiation efficiency over these experiments was 67.7%.

RNA, triglycerides, and metabolites were collected from each batch of differentiated cells. Expression of *ADIPOQ* and accumulation of triglycerides correlated with differentiation efficiency (Figures 2D and 2E). Although the differentiation efficiency of some cell lines was below the mean value of 67%, all cell lines expressed *FABP4* following doxycycline treatment (Figure S2D). *FABP4* is a direct transcriptional target of PPARG2, indicating viral transduction and activity of transgenic PPARG2 even in poorly differentiated cell lines.

Directed Differentiation of iPSCs into Hepatocyte-like Cells

We used fluorescence-activated cell sorting (FACS) to prospectively isolate HLCs derived by a directed differentiation protocol (Peters et al., 2016). As with the differentiation of white adipocytes, each iPSC line was differentiated twice to account for variability, resulting in 136 HLC differentiations (Figure 1A). Using asialoglycoprotein receptor 1 (ASGR1) as a surface marker of hepatocytes, we purified HLCs from the heterogeneous population of cells produced by differentiation through definitive endoderm (DE), hepatic endoderm (HE), immature hepatocytes (IMHs), and, finally, into mature hepatocytes (MHs) (Figure 2F). HLCs express albumin and ASGR1 (Figure 2G) and perform lipid processing in a biologically relevant fashion responsive to perturbations of sortilin expression (Ding et al., 2013). Differentiation efficiencies for each of the 68 iPSC lines was measured by ASGR1 FACS (Figure 2H) and varied from 65% to 0.6%. The mean and median differentiation efficiencies were 13% and 9.9%, respectively.

Expression of the hepatocyte markers *ALB* and *APOB* by FACS-isolated HLCs was analyzed by qRT-PCR (Figures 2I

and 2J). Nearly all isolated HLC populations expressed *ALB* and *APOB*, and the expression levels of these two genes were positively correlated (Pearson correlation coefficient $[r] = 0.48$). In a minority of cell lines where differentiation efficiency was very low, the expression of *ALB* and *APOB* was similarly diminished, equivalent to expression of the negative control gene *CD4*. Sorting of non-specific cells was diminished by the use of an isotype control antibody for each FACS replicate (Figure S2E). For modeling of the lipid phenotype associated with the 1p13 genotype of these cells, conditioned medium and metabolites were extracted from adherent unsorted HLCs.

Modeling of the eQTL Occurring at 1p13 Accurately Recreates the In Vivo Phenotype

The minor haplotype at the 1p13 locus has been associated with decreased plasma LDL-C concentrations and a decreased risk of MI, mediated by increased hepatic sortilin expression (Musunuru et al., 2010). Three genes (*CELSR2*, *PSRC1*, and *SORT1*) are upregulated in hepatocytes through creation of a putative C/EBP transcription factor binding site by the causal SNP (rs12740374; Figure 3A). In vitro modeling of the hepatic eQTL at this locus accurately reproduced the previously published expression profile from liver biopsies, with *CELSR2*, *PSRC1*, and *SORT1* increased in ASGR1-positive HLCs carrying the minor 1p13 locus haplotype compared with matched cells carrying the major 1p13 locus haplotype (Figure 3B). Although significant ($p = 0.04$), the increase in *SORT1* expression is modest (1.3-fold). Expression of *CELSR2*, *PSRC1*, and *SORT1* does not appear to correlate with differentiation efficiency (Figures S3D–S3F) because FACS isolation of ASGR1-positive HLCs eliminates nearly all undifferentiated cells (isotype controls are used to gate out undifferentiated cells). In contrast, white adipocytes demonstrated no change in *SORT1* expression between the major and minor haplotype (Figure 3D), consistent with previously published data from primary adipose tissue samples (Figure 3D), and *SORT1* expression did not correlate with differentiation efficiency in white adipocytes (Figure S3G).

To validate our experimental findings, we used clustered regularly interspaced palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) genome editing techniques to knock out the causal SNP rs12740374 in a cell line carrying two minor alleles at the 1p13 locus (Figure S3A). The resultant knockout cell lines carried deletions of rs12740374 and surrounding base pairs as their only genetic difference from the isogenic parental and wild-type cell lines. Each mutant cell line incurred homozygous deletion of the rs12740374 base pair with heterozygous deletions of the surrounding base pairs. The largest deletion included 27 base pairs and the entire putative C/EBP transcription factor binding site, and the smallest deletion removed three base pairs and inserted three base pairs, leaving four base pairs of the putative C/EBP transcription factor binding site. In each case, the rs12740374 base pair was deleted, removing the critical thymine in the canonical C/EBP transcription factor binding site created by the minor allele of this SNP. We differentiated these four knockout clones and two wild-type clones into HLCs and isolated ASGR1-positive cells by FACS. In this experiment, we observed a 1.3-fold increase in expression of *SORT1* in ASGR1-positive HLCs derived

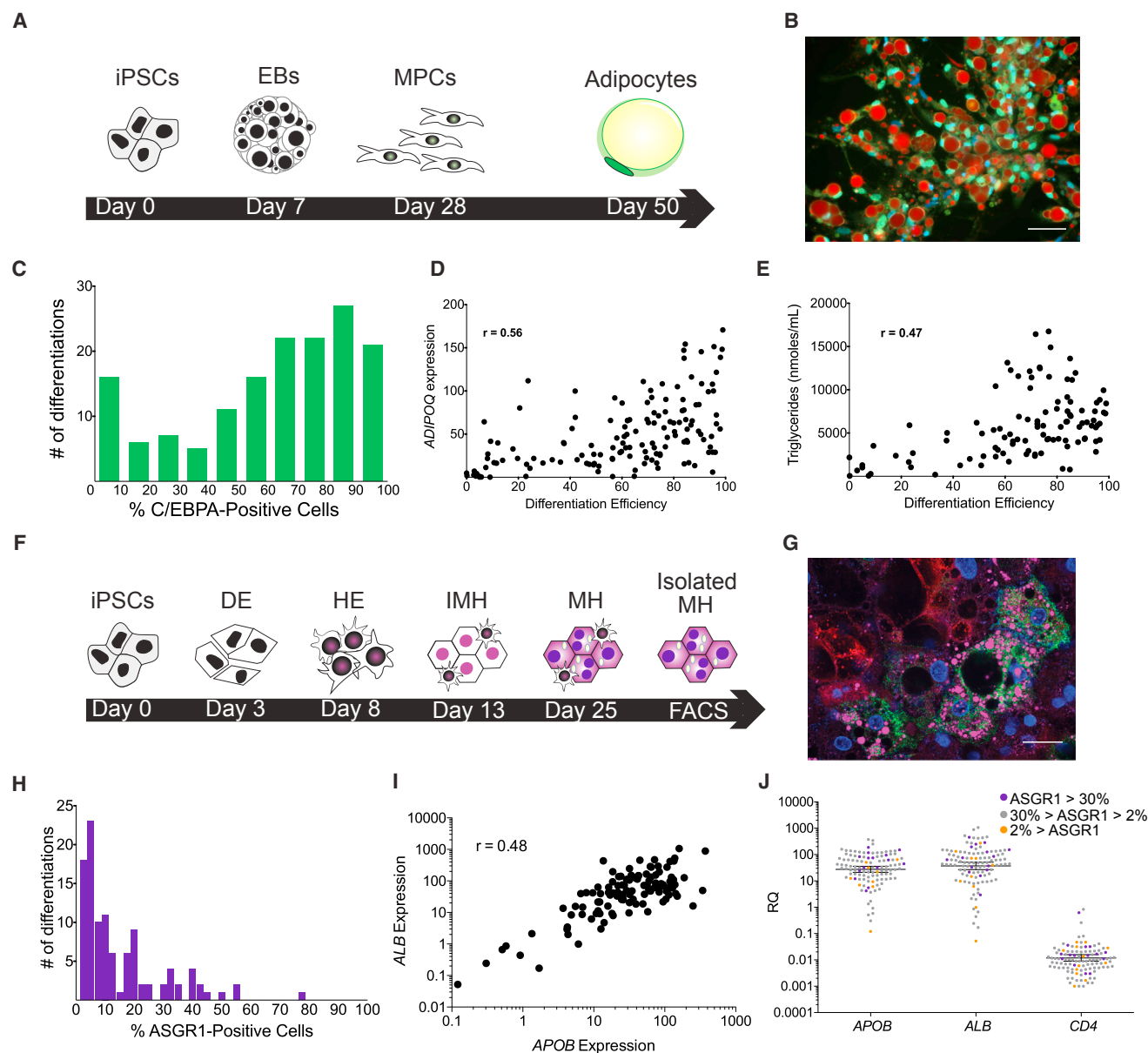


Figure 2. Differentiation of iPSCs into Adipocytes and HLCs

(A) Differentiation protocol for production of white adipocytes.

(B) Immunofluorescence indicating expression of C/EBP- α (green) and accumulation of triglycerides (red). Nuclei, blue. Scale bar, 100 μ m.

(C) Differentiation efficiencies recorded for each differentiation experiment.

(D and E) Expression of *ADIPOQ* correlates with differentiation efficiency (D, $n = 136$), as does the accumulation of triglycerides (E, $n = 90$).

(F) Directed differentiation protocol for production and isolation of HLCs.

(G) Immunofluorescence indicating expression of the hepatocyte markers ASGR1 (green) and albumin (red) as well as accumulation of triglycerides (pink). Hoechst, blue. Scale bar, 50 μ m.

(H) Differentiation efficiencies for each iPSC line as measured by flow cytometry for surface ASGR1 expression ($n = 136$).

(I) Correlation of *ALB* and *APOB* expression in ASGR1-positive HLCs ($n = 136$).

(J) qRT-PCR data indicating expression of *APOB* and *ALB* and non-existent expression of *CD4* (as a negative control) ($n = 136$). Line, mean; bars, SEM. RQ as described in the STAR Methods.

See also Figure S2.

from the minor haplotype parental cells compared with the genome-edited clonal cell lines (Figure 3C). This finding matched precisely that of the in vitro eQTL that we demonstrated in the cohort of iPSC-derived hepatocytes (Figure 3B). Both the

genome-edited clonal cell lines and parental cell lines demonstrated more than 10% differentiation efficiency prior to FACS (Figure S3B), and there was no difference in *ALB* expression between these cell lines after sorting (Figure S3C).

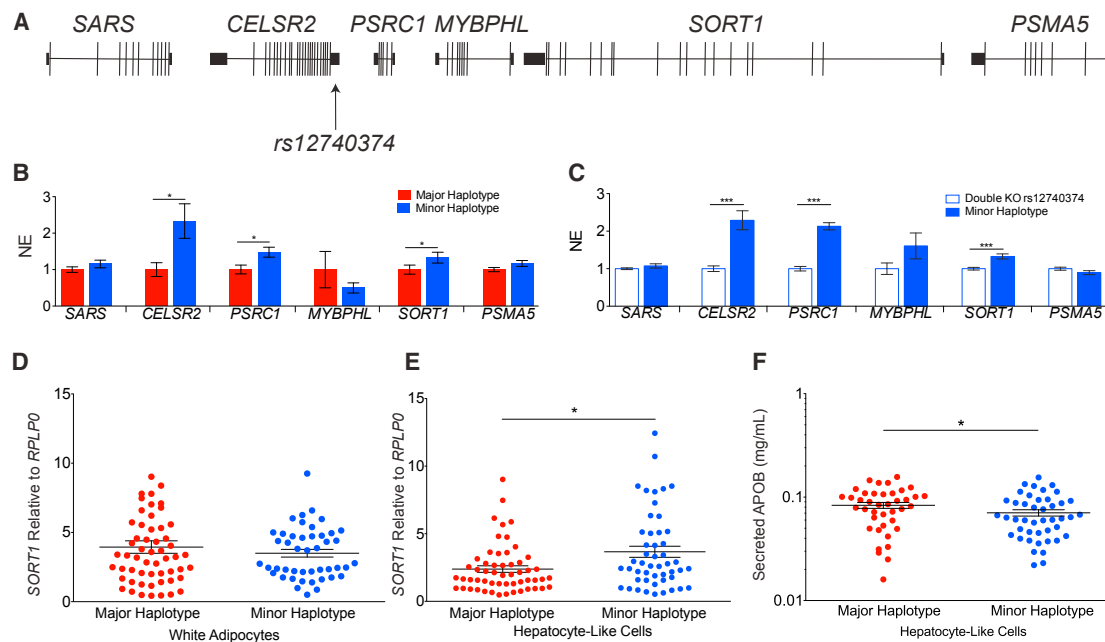


Figure 3. Phenotyping of Differentiated HLCs and Adipocytes Demonstrates In Vitro Recapitulation of the 1p13 *SORT1* eQTL

(A) The 1p13 locus encodes six genes in linkage disequilibrium, and the causal SNP (rs12740374) associated with decreased serum LDL cholesterol and increased expression of three genes is located in the 3' UTR of *CELSR2*.

(B) qRT-PCR analysis of ASGR1-positive HLCs homozygous for the major and minor haplotypes at 1p13 (n = 68). NE, normalized expression, normalized to major genotype. *CELSR2* p = 0.016, *PSRC1* p = 0.011, *SORT1* p = 0.046, Student's t test.

(C) qRT-PCR analysis of ASGR1-positive genome-edited HLCs (n = 18 wild-type, n = 20 mutant). NE, normalized expression, normalized to minor genotype. *CELSR2*, *PSRC1*, and *SORT1* p values < 0.001, unpaired t test.

(D) Scatterplot of qRT-PCR for *SORT1* in differentiated adipocytes homozygous for the major or minor haplotype at the 1p13 locus (n = 68).

(E) Scatterplot of qRT-PCR for *SORT1* in ASGR1-positive HLCs homozygous for the major or minor haplotype at the 1p13 locus (n = 68). p = 0.019, Student's t test.

(F) Scatterplot of APOB accumulation in HLC-conditioned medium as measured by ELISA. p = 0.0258, Student's t test.

Line in scatterplots, mean; bars in all plots, SEM. See also Figure S3.

Apolipoprotein B (APOB) secretion has been used as an accurate surrogate of LDL secretion in vitro. We collected conditioned medium from differentiated cells and performed an ELISA to measure the effect of 1p13 genotype on HLC secretion of APOB. HLCs carrying the minor genotype secreted significantly (30%) less APOB than major genotype HLCs (Figure 3F). This is consistent with previous findings from human serum (Musunuru et al., 2010). APOB secretion correlated with differentiation efficiencies for each cell line (Figure S3H), and there was no statistical difference in HLC differentiation efficiency between cells carrying the major or minor genotypes (Figure S3I).

Cell Transcriptomes Accurately Recreate In Vivo Phenotypes and the 1p13 eQTL

RNA sequencing analyses were performed on samples from iPSCs, white adipocytes, and ASGR1-positive HLCs from all 68 cell lines. Cellular identity and *SORT1* expression related to the 1p13 genotype were the focus of the preliminary analysis of these datasets. The transcriptional profile of each cell type was distinct, as indicated by transcriptome-wide heatmaps (Figure S4A). Tissue-specific biological processes were upregulated in each cell type, as evidenced by gene ontology analysis (Figures S4B–S4D). iPSCs, white adipocytes, and HLCs all cluster separately in a principal-component analysis (PCA) analysis of

their gene expression profile (Figure S4E), suggesting that differentiation produced transcriptionally distinct cell types. Cell type-specific gene expression results obtained by qRT-PCR were also replicated in the RNA sequencing dataset (Figure S4F). The gene expression profiles of clonal cell lines derived from the same donor correlated tightly (Figure S4G).

1p13 *SORT1* eQTL modeling was replicated in the RNA sequencing (RNA-seq) dataset by applying a linear mixed model analysis using data from iPSCs, white adipocytes, and HLCs. The predicted eQTL was replicated accurately in all five genes of the 1p13 locus, with *PSRC1*, *CELSR2*, and *SORT1* each being statistically upregulated in minor genotyped HLCs (Figure 4A). *PSRC1* was also upregulated in minor genotyped adipocytes and iPSCs. As expected, there was no expression phenotype at either *SARS* or *PSMA5* in any cell type. This phenotype was also corroborated when analyzing the number of fragments per kilobase per million reads (FPKM), an alternative measure of transcript abundance (Figure 4B). This result is evidence that iPSCs can be used through directed differentiation to model phenotypes driven by common, non-coding variants.

In addition to the chromosome 1p13 eQTL findings presented in Figure 4A, a number of novel *trans*-eQTLs were identified in HLCs (Table S2). Included are the genes that were below the false discovery rate of 10%. No gene ontology process terms

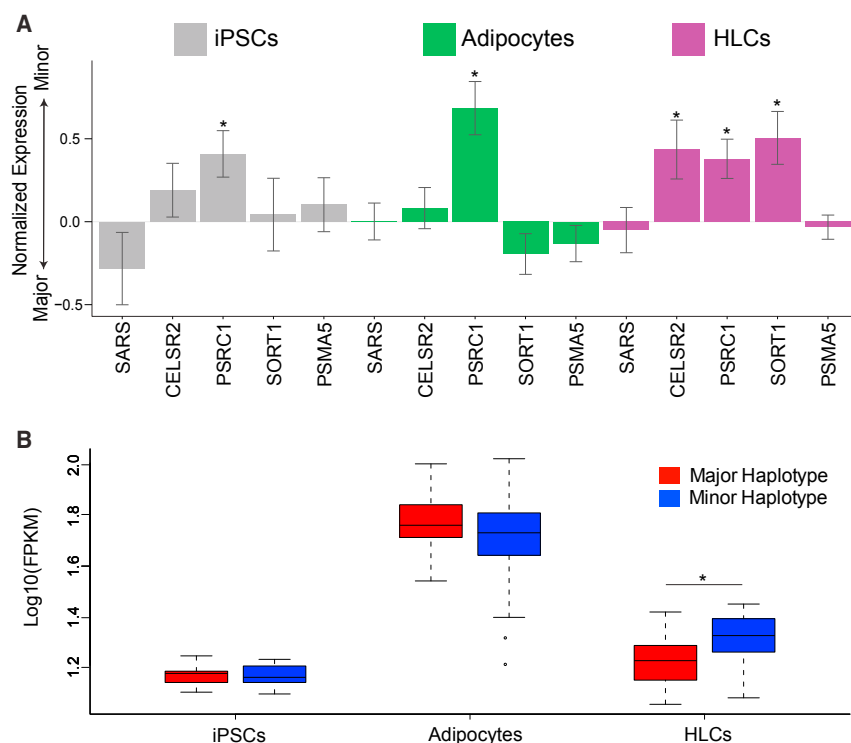


Figure 4. RNA Sequencing Analyses Support Accurate Modeling of the 1p13 *SORT1* eQTL

(A) RNA sequencing data comparing expression of genes in the chromosome 1p13 linkage disequilibrium block in cells carrying the major or minor haplotype. Values above and below the x axis display increased expression in cells carrying the minor or major haplotype, respectively (n = 68). FDR values are summarized in Table S3.

(B) *SORT1* expression in iPSCs, adipocytes, and HLCs is measured as FPKM. Bars, mean; whiskers, SD. Outliers in adipocyte data are plotted as dots. p Value from HLCs = 0.034, Student's t test. See also Figure S4.

were enriched in this gene set. There were no *trans*-eQTLs identified in either iPSCs or adipocytes.

Encouraged by the clear association of the 1p13 genotype with *SORT1* expression in our dataset, we replicated additional eQTL analyses (Table S4). 50 loci previously identified by eQTL analysis in liver tissue (Schadt et al., 2008; Teslovich et al., 2010) were tested for their effect on gene expression. Two SNPs (rs2929282 and rs3177928) correlated with expression of *FRMD5* and *HLA-DRB1* with false discovery rates (FDRs) of 9.5% and 3.3%, respectively. rs10889353 and rs10195252 correlated with expression of *DOCK7* and *GRB14*, respectively (both at an FDR of 11.6%). The other 46 SNPs were not significantly correlated with gene expression in HLCs.

Metabolomics Analyses Indicate a General Downregulation of Lipid Accumulation in Minor Genotyped HLCs

Intracellular metabolite extraction was performed as described previously by our group (Patsch et al., 2015). Metabolomic profiling was performed on all three cell types using two targeted profiling methods. The first method was run in positive ion mode and concentrated on lipid subspecies. The second method, the Amide method, was run in negative ion mode and focused on nucleotide and nucleoside phosphates, high-energy intermediates, organic acids, tricarboxylic acid (TCA) cycle intermediates, and glycolytic intermediates. Both methods indicate distinct metabolic profiles for each of the three cell types, as indicated by PCA plots (Figures S5A and S5B).

In both iPSCs (Figure S5C) and adipocytes (Figure S5D), there were no significant differences in lipid metabolites between the minor and major genotype. In iPSCs, the Amide method revealed no significant differences between the major and minor genotype

(Figure S5E). In adipocytes, there was a significant decrease in the levels of the nucleoside cytidine diphosphate (CDP) (FDR < 5%) in the minor genotype and a significant increase in the levels of the ubiquitous organic aminosulfonic acid taurine (FDR < 5%) in the minor genotype (both are indicated in green in Figure S5F).

In the HLCs, there were many metabolites with significant differences between minor and major genotypes (indicated in purple in Figures 5A and 5B). The greatest

differences were seen in the lipid metabolites, with some species enriched up to 4-fold in major genotype HLCs versus minor genotype cells. The minor genotype HLCs accumulated significantly less phosphatidylethanolamine, triacylglycerol, diacylglycerol, phosphatidylcholine, and sphingomyelin species (FDR < 5%; Figure 5B, indicated in blue), suggesting a non-specific downregulation of lipid synthesis in the minor genotype HLCs. Each bar in Figure 5B represents a subspecies of lipid metabolite; e.g., PC 36:2, where 36 is the number of carbons and 2 is the number of double bonds. Genotype-driven lipid dysregulation occurs across a wide range of subspecies within each lipid class. Minor genotype HLCs also retained significantly less acetoacetic acid (FDR < 5%; Figure 5C), a ketone body whose main function is to provide acetoacetyl coenzyme A (CoA) and acetyl CoA for synthesis of cholesterol, fatty acids, and complex lipids. 2-Aminoadipic acid, a metabolite known to be associated with cardiometabolic disease (Wang et al., 2013), was also significantly decreased in minor genotype HLCs (FDR < 5%; Figure 5C), as were D-gluconic acid, kynurenine, and pseudouridine (FDR < 5%; Figure 5C).

DISCUSSION

Community-based cohorts have increased the power of human genetics, rendering possible the identification of many novel disease loci. GWAS and eQTL analyses overcame the limitations of traditional human genetics by coupling very large participant cohorts with relatively inexpensive whole-genome genotyping chips and clinical data regarding markers of disease. In this way, subtle effects of common variants were extracted from the noise of genome-wide variation (Teslovich et al., 2010).

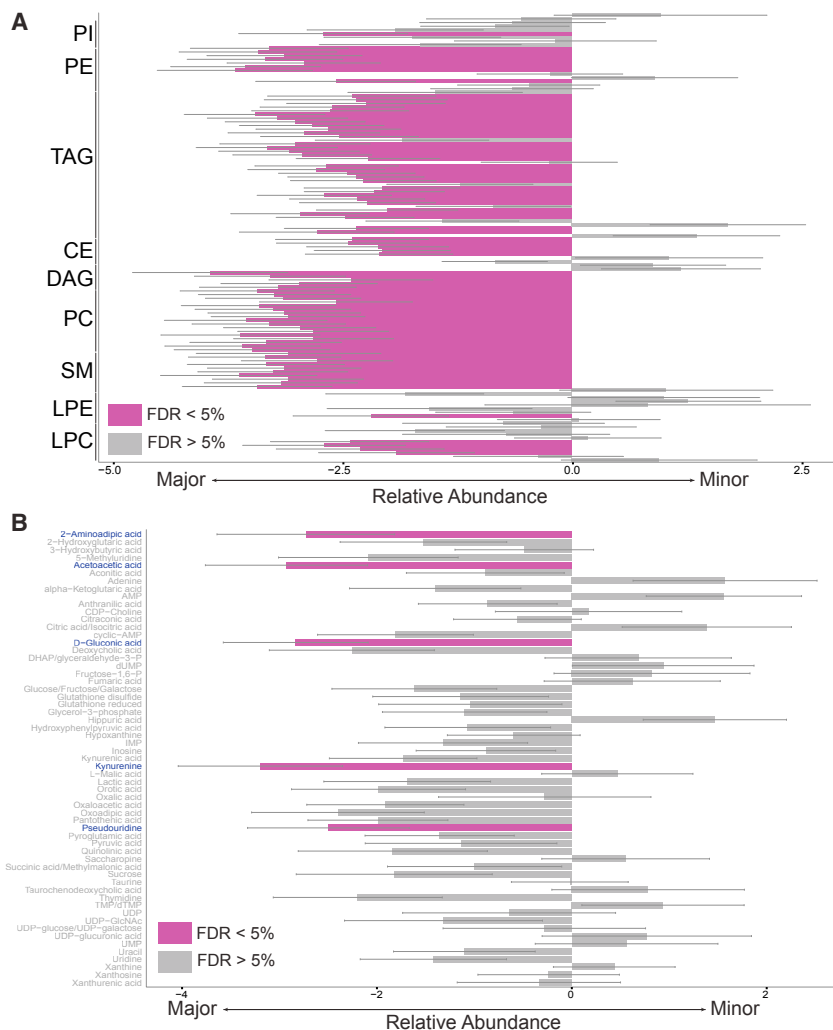


Figure 5. Metabolomic QTL Findings in Differentiated HLCs

(A and B) Lipid (A) and Amide metabolomics (B) profiling of HLCs. Analyte classes are indicated on the y axis. For legibility, only the lipid classes are labeled in the y axis, and each bar represents a subspecies of lipid within the larger lipid class. Subspecies are labeled in full in parallel charts [Figures S5C and S5D](#). Bars to the left or right of the y axis indicate enrichment in cells carrying the major or minor haplotype at chromosome 1p13, respectively. Values that satisfy the 5% FDR rate are highlighted in purple. FDR values are summarized in [Table S3](#). PI, phosphatidylinositols; PE, phosphatidylethanolamines; TAG, triacylglycerols; CE, cholesteryl ester; DAG, diacylglycerols; PC, phosphatidylcholines; SM, sphingomyelins; LPC, lysophosphatidylcholines; LPE, lysophosphatidylethanolamines. $n = 408$. See also [Figure S5](#).

certained at the rs12740374 locus were differentiated into HLCs and accurately recapitulated gene expression profiles in genes centered on the 1p13 locus. For future in vitro eQTL analyses studying hepatic eQTLs, the *SORT1* phenotype will be useful as a positive control, given that the cohort is large enough and includes participants homozygous for the major and minor haplotypes at chromosome 1p13. iPSCs and cognate adipocytes demonstrated no effect of genotype on expression of *SORT1*, in keeping with in vivo findings.

It is important to note that the effect size of genotype on hepatic gene expression at

1P13, as measured by qRT-PCR, is smaller than that measured previously measured in primary tissues by qRT-PCR and cDNA microarray in primary tissues. It is possible that this discrepancy in effect size is due to the relative “immaturity” of HLCs in comparison with primary hepatocytes ([Si-Tayeb et al., 2010](#)) or due to heterogeneity in differentiation. Differentiation of the iPSC cohort and generation of isogenic genome-edited knockout clones of rs12740374 resulted in an identical effect size on *CELSR2*, *PSRC1*, and *SORT1* gene expression. This suggests that the cohort size we have assembled is adequate to accurately perform this particular eQTL analysis in vitro but that there is some aspect of in vitro culture or incomplete HLC maturation that dampens the transcriptional effect of this SNP.

Creation of isogenic genome-edited cell lines in the manner presented here is made possible by previous mechanistic investigation identifying the causal SNP at this locus. In contrast, the in vitro study of other GWAS or eQTL loci will continue to rely on large cohorts of cell lines until their respective causal SNPs are identified. At this time, no genome editing technology is capable of replacing large tracts of genomic DNA, effective in swapping linkage disequilibrium haplotypes in otherwise isogenic backgrounds. Until such a development or identification of causal

Simultaneously, the advent of iPSC technology increased the utility of cellular disease models for the study of genotype-phenotype interactions ([Takahashi et al., 2007](#)). The capacity to generate pluripotent cells from the somatic cells of any participant or patient of interest provides a direct insight into the relationship between that individual’s genotype and the functional capacity of the differentiated cells ([Park et al., 2008](#)). Here, for the first time, we have demonstrated that iPSC technology may be feasibly applied to in vitro genetics, given the appropriate cohort size and a carefully planned experiment. This study design eliminates a limitation of the traditional GWAS or eQTL analysis: availability of differentiated cells for mechanistic study of novel disease-associated loci. The advantages of such future in vitro population genetics studies would be manifold. Most notably, future in vitro studies would make possible the correlation of cellular and subcellular phenotypes with genome-wide variation, especially in cell types that are difficult to procure in large quantities from primary tissues, such as neurons or β cells.

We have modeled the association between rs12740374 and hepatic expression of *SORT1*, previously reported to protect from MI secondary to decreased circulating LDL-C concentrations ([Musunuru et al., 2010](#)). iPSCs derived from participants as-

SNPs, discovery of and mechanistic investigation into the effect of common genetic variants on cellular phenotypes will be made possible by the use of large iPSC cohorts as presented here.

Although iPSCs and adipocytes lacked *trans*-eQTLs, HLCs also demonstrated a number of *trans*-eQTLs, potentially as downstream effects of decreased lipid accumulation or as direct effects of increased *SORT1* expression. One potential advantage of an in vitro study of common genetic variants is the removal of contaminating cell types from tissue samples, purifying the cell type-specific effect of genotype on gene expression and functional outputs. Although the cohort sizes required for GWASs are orders of magnitude larger than that of this study, we observed that the cohort size of 34 major and 34 minor cell lines was sufficient to accurately model both transcriptional and metabolic effects of this common variant.

Although we assembled this cohort based upon genotype at the rs12740374 SNP, we performed follow-up eQTL analyses on 50 SNPs previously associated with gene expression signatures (Schadt et al., 2008; Teslovich et al., 2010). Four of these loci were marginally significant in correlation with gene expression; *DOCK7*, *GRB14*, *FRMD5*, and *HLA-DRB1* correlated with genotype at rs10889353, rs10195252, rs2929282, and rs3177928, respectively (Table S4). These associations were discovered despite the fact that this experiment was underpowered to perform eQTL analysis at loci other than 1p13. Given a larger cohort size, we expect that many additional eQTL analyses and follow-up functional studies will be made possible in HLCs.

Utilizing two metabolomics platforms, we performed intracellular metabolite profiling of iPSCs, adipocytes, and HLCs. Lipid metabolite profiles were dramatically decreased in HLCs carrying the minor 1p13 haplotype but not in cognate iPSCs or adipocytes. The significantly decreased lipid species included phosphatidylethanolamines, triacylglycerols, diacylglycerols, phosphatidylcholines, and sphingomyelins. Further, the results from the Amide metabolite detection platform (a method to quantify predominantly organic acids and similar small molecules) demonstrated a significant reduction of acetoacetic acid in the minor genotype HLCs. The primary function of this ketone body is to provide acetoacetyl CoA and acetyl CoA for synthesis of cholesterol, fatty acids, and complex lipids. These results suggest a globally decreased synthesis of lipid species and a faithful reproduction, in an in vitro cellular model, of the lipid changes seen in the sera of patients with the same hepatic *SORT1* eQTL.

2-aminoadipic acid, a product of lysine degradation, was significantly decreased in minor genotype HLCs. This metabolite has previously been identified by our group as a mediator of cardiometabolic risk (Wang et al., 2013), and it is intriguing that it is decreased in HLCs that carry an eQTL associated with reduced lipid levels. Other metabolomic findings generate more questions than answers. For example, D-gluconic acid, an oxidative product of glucose, was also significantly decreased in minor genotype HLCs. So was kynurenine, a product of tryptophan metabolism and a precursor of niacin, and pseudouridine, a modified nucleoside, but the relevance of these findings is not immediately clear.

These metabolic findings, in addition to the *trans*-eQTLs identified in HLCs, may point toward previously uncharacterized underlying biological mechanisms of MI and other forms of athero-

sclerotic cardiovascular disease (CVD). One suggestive finding from the *trans*-eQTL gene set is the 3-fold downregulation of *PLTP* (previously associated with plasma lipid concentrations by GWASs) in HLCs carrying the minor 1p13 haplotype compared with major haplotyped HLCs. *PLTP* has been implicated in lipoprotein assembly in hepatocytes (Manchekar et al., 2015; Yazdanyar and Jiang, 2012; Yazdanyar et al., 2013) in a directional manner consistent with our current findings. It is possible that *PLTP* downregulation may affect or contribute to the generally decreased accumulation of lipids in minor haplotype HLCs. Other genes in this dataset have been associated by GWASs with CVD-relevant traits, including *EYS*, *PCDHA9*, *MFSD4B*, *CYB561D1*, *PCDHA10*, *CPN1*, and *SNX5*. Among these genes, *CYB561D1*, *PLTP*, *CPN1*, *CPNE1*, and *SNX5* are expressed in normal human hepatocytes. Future mechanistic studies will delineate the contribution of *PLTP* and these other genes identified by *trans*-eQTL in the relationship between 1p13 haplotype and CVD pathogenesis. Potential mechanistic discoveries of this kind are one of the advantages of an iPSC-driven study of common genetic variants, enabled by the abundance of cellular material made possible by differentiation and the depletion of contaminating cell types prevalent in primary samples.

This study serves as proof of concept that in vitro eQTL analyses are indeed possible using iPSC technology and will facilitate more ambitious population genetics analyses performed in the dish. It is our expectation that improvements in differentiation protocols and reprogramming strategies will make this strategy more accurate and commonplace. Future studies with larger cohorts could be used for functional quantitative trait locus (fQTL) analyses, correlating common genetic variants with cellular functions. Such studies may provide significant incremental functional genomic information regarding tissue-specific mechanisms of disease, thereby extending the utility of traditional QTL and GWAS designs.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures, four tables, and one dataset and can be found with this article online at <http://dx.doi.org/10.1016/j.stem.2017.01.010>.

AUTHOR CONTRIBUTIONS

C.R.W. and J.F.O. designed and performed experiments, analyzed data, and prepared the manuscript. M.F. designed and performed experiments. C.E.B. and D.T.P. designed and performed experiments and analyzed data. A.M.T., X.Z., and X.S. analyzed data. J.E.M. performed and analyzed experiments. P.L., Y.W., J.C., F.X., M.H.C.F., E.D., L.C., and J.S. performed experiments. R.C.D. analyzed data and prepared the manuscript. X.J. designed experiments. K.M. supported D.T.P., A.M. supported A.M.T. S.K., L.D., J.Z., R.E.G., R.S.V., and C.J.O. supported and designed the study. C.A.C. designed experiments, prepared the manuscript, and supported the study.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
FG-purified Anti-Human CD3, Clone: OKT3	eBioscience	Cat#160037-81; RRID:AB_468854
OCT4	Abcam	Cat#ab19857; RRID: AB_445175
NANOG	Abcam	Cat#ab21624; RRID: AB_446437
SSEA3	Millipore	Cat#MAB4303; RRID: AB_177628
SSEA4	Millipore	Cat#MAB4304; RRID: AB_177629
Tra-1-60	Millipore	Cat#MAB4360; RRID: AB_2119183
C/EBP-alpha	Abcam	Cat#ab40761; RRID: AB_726792
Albumin	Bethyl Laboratories	Cat#E80-129; RRID: AB_1286522
ASGR1	Becton Dickinson Biosciences	Cat#563654
ASGR1-PE	Becton Dickinson Biosciences	Cat#563655
Mouse IgGκ Isotype Control	Becton Dickinson Biosciences	Cat#555749; RRID: AB_396091
Biological Samples		
Human Blood Samples	Framingham Heart Study Offspring Cohort	N/A
Chemicals, Peptides, and Recombinant Proteins		
Human Recombinant Interleukin 2	Becton Dickinson	Cat#354043
Collagenase IV	Stem Cell Technologies	Cat#07909
CHIR99021	Cayman Chemical	Cat#13122-10mg
Activin A	Cell Guidance Systems	Cat#GFH6-1000
Bone Morphogenetic Protein 4	R&D Systems	Cat#314-BP
Hepatocyte Growth Factor	Peptotech	Cat# 100-39
Dimethyl Sulfoxide	Sigma-Aldrich	Cat#D2650
Oncostatin M	Peptotech	Cat#300-10
Dexamethasone	Sigma-Aldrich	Cat#D1756
Rosiglitazone	Santa Cruz	Cat#sc-202795
Insulin	Sigma-Aldrich	Cat#I9278
Basic Fibroblast Growth Factor	Aldevron	Cat#7001
Critical Commercial Assays		
Cytotune-iPS 2.0 Sendai Reprogramming Kit	Thermo Fisher	Cat# A16517
RNeasy Mini Kit	QIAGEN	Cat# 74104
High-Capacity cDNA Reverse Transcription Kit	Thermo Fisher	Cat#4368814
384-well TaqMan hPSC Scorecard kits	Thermo Fisher	Cat#A15872
DC protein assay	Bio-Rad Laboratories	Cat# 5000116
Infinity Triglycerides Reagent	VWR	Cat# 46100-346
Human APOB ELISAPro kit	Mabtech	Cat# 3715-1HP-2
GLOBINclear Kit, human, for globin mRNA depletion	Thermo Fisher	AM1980
TruSeq RNA Library Prep Kit v2	Illumina	Cat#RS-122-2001
Deposited Data		
RNA Sequencing data	NCBI Gene Expression Omnibus	GEO: GSE90749
Experimental Models: Cell Lines		
69 iPSC lines	This Study	N/A
rs12740374 knockout clones	This Study	N/A
Recombinant DNA		
FU-TetO-Gateway	Addgene	Cat#43914

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Lenti-Doxycycline-PPARG2	(Ahfeldt et al., 2012; Lee and Cowan, 2014)	N/A
FUdeltaGW-rtTA	Addgene	Cat#19780
pRSV-Rev	AddGene	Cat#12253
pMDLg/pRRE	AddGene	Cat#12251
pCas9_GFP	Addgene	Cat#44719
gRNA cloning Vector	Addgene	Cat#41824
Sequence-Based Reagents		
RPLPO Forward Primer (5' to 3'): GCAGCATCTACAACCCTGAAG	This Study	N/A
RPLPO Reverse Primer (5' to 3'): GCAGACAGACACTGGCAACA	This Study	N/A
DNMT3B Forward Primer (5' to 3'): ATAAGTCGAAGGTGCGTCGT	This Study	N/A
DNMT3B Reverse Primer (5' to 3'): GGCAACATCTGAAGCCATTT	This Study	N/A
hTERT Forward Primer (5' to 3'): TGTGCACCAACATCTACAAG	This Study	N/A
hTERT Reverse Primer (5' to 3'): GCGTTCTTGGCTTTCAGGAT	This Study	N/A
NANOG Forward Primer (5' to 3'): CAGTCTGGACACTGGCTGAA	This Study	N/A
NANOG Reverse Primer (5' to 3'): CTCGCTGATTAGGCTCCAAC	This Study	N/A
hTERT Forward Primer (5' to 3'): TGTGCACCAACATCTACAAG	This Study	N/A
hTERT Reverse Primer (5' to 3'): GCGTTCTTGGCTTTCAGGAT	This Study	N/A
OCT4 Forward Primer (5' to 3'): TGTACTCCTCGTCCCTTTC	This Study	N/A
OCT4 Reverse Primer (5' to 3'): TCCAGGTTTTCTTCCCTAGC	This Study	N/A
REX1 Forward Primer (5' to 3'): TGGACACGTCTGTGCTCTTC	This Study	N/A
REX1 Reverse Primer (5' to 3'): GTCTTGGCGTCTTCTCGAAC	This Study	N/A
SOX2 Forward Primer (5' to 3'): GCTAGTCTCCAAGCGACGAA	This Study	N/A
SOX2 Reverse Primer (5' to 3'): GCAAGAAGCCTCTCCTTGAA	This Study	N/A
NT5E Gene Expression Assay	Thermo Fisher	Cat#Hs00159686_m1
CD44 Gene Expression Assay	Thermo Fisher	Cat#Hs01075861_m1
ENG Gene Expression Assay	Thermo Fisher	Cat#Hs00923996_m1
CD4 Gene Expression Assay	Thermo Fisher	Cat#Hs01058407_m1
ALB Gene Expression Assay	Thermo Fisher	Cat#Hs00910225_m1
ADIPOQ Gene Expression Assay	Thermo Fisher	Cat#Hs00605917_m1
FABP4 Gene Expression Assay	Thermo Fisher	Cat#Hs01086177_m1
APOB Gene Expression Assay	Thermo Fisher	Cat#Hs01071209_m1
SARS Gene Expression Assay	Thermo Fisher	Cat#Hs00197856_m1
CELSR2 Gene Expression Assay	Thermo Fisher	Cat#Hs00154903_m1
PSRC1 Gene Expression Assay	Thermo Fisher	Cat#Hs00934027_g1
MYBPHL Gene Expression Assay	Thermo Fisher	Cat#Hs00975401_m1
SORT1 Gene Expression Assay	Thermo Fisher	Cat#Hs00361760_m1
PSMA5 Gene Expression Assay	Thermo Fisher	Cat#Hs00936004_m1
RPLP0 Gene Expression Assay	Thermo Fisher	Cat#326314E
rs12740374 Genotyping F (5' to 3'): AGTTGCTGACCCAAAAGTGC	This Study	N/A
rs12740374 Genotyping R (5' to 3'): GAGGCCACAGCAGGTTAGAC	This Study	N/A
Software and Algorithms		
GraphPad Prism	Graphpad Software	N/A
Bowtie2	(Kim et al., 2013)	http://bowtie-bio.sourceforge.net/bowtie2/index.shtml
Cufflinks	(Roberts et al., 2011)	http://cole-trapnell-lab.github.io/cufflinks/
Subread package for R	(Liao et al., 2014)	http://subread.sourceforge.net/

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
PEER package for R	(Stegle et al., 2012)	http://www.sanger.ac.uk/science/tools/peer
Multiquant Software	Sciex	https://sciex.com
GenABEL package for R	(Aulchenko et al., 2007)	https://cran.r-project.org/web/packages/GenABEL/index.html
Other		
Vacutainer CPT Cell Preparation Tubes with Sodium Citrate, 4 mL draw capacity	Becton Dickinson	Cat#362760
X-VIVO 10 with Gentamicin and Phenol Red, 1 L	Lonza	Cat#04-380Q
5% Type AB Heat Inactivated Human Serum	Valley Biomedical	Cat#HP1022HI
mTeSR 1	Stem Cell Technologies	Cat#05850
Gentle Cell Dissociation Reagent	Stem Cell Technologies	Cat#07174
Knockout Serum Replacement	Thermo Fisher	Cat#10828010
LipidTox Red and LipidTox Deep Red dyes	Thermo Fisher	Cat# H34476 and H34477
Taqman Gene Expression Master Mix	Thermo Fisher	Cat# 4369016
FAST SYBR Green Master Mix	Thermo Fisher	Cat# 4385616
Accutase	Stem Cell Technologies	Cat# 07920
Y-27632	Stem Cell Technologies	Cat# 72302
Methanol (Optima LC/MS)	Fisher Chemical	Cat#A456-1
2-Propanol (Optima LC/MS)	Fisher Chemical	Cat#A461-1
Water (HPLC)	Fisher Chemical	W5-4
Chloroform (HPLC)	Fisher Chemical	C607-1
L-Phenylalanine- ¹³ C ₉ , ¹⁵ N, $\alpha,\beta,\beta,2,3,4,5,6$ -d ₈	Sigma Aldrich	Cat#749885
L-Valine-d ₈	Cambridge Isotope Laboratories	Cat# DLM-7784-PK
Acetonitrile, HPLC	J.T. Baker	Cat# 9012-03
XBridge BEH Amide Column, 130Å, 3.5 μ m, 4.6 mm X 30 mm, 1/pkg	Waters	Cat#186004866
Ammonium Acetate	Sigma Aldrich	Cat#17836-50G
Ammonium hydroxide solution	Sigma Aldrich	Cat#17837

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for reagents may be directed to, and will be fulfilled by, the Lead Contact, Chad Cowan (chad_cowan@harvard.edu).

EXPERIMENTAL MODELS AND SUBJECT DETAILS

iPSC lines were derived from participants of the FHS Offspring Cohort. For details concerning the sex and karyotype of each cell line, refer to [Table S1](#). Informed consent was obtained from all participants under Institutional Review Board Approval at both Boston University and Harvard University. All iPSC lines will be made available through WiCell in line with standard policies for academic and corporate users.

METHOD DETAILS**Peripheral Blood Mononuclear Cell (PBMC) Isolation**

Fasting blood specimens were collected on the morning of PBMC isolation. Samples were placed in Vacutainer® CPT Cell Preparation Tubes with Sodium Citrate, 4 mL Draw Capacity (BD) with PBS added to a volume of 3.5 mL, and centrifugation and cell washing steps were performed according to the manufacturer's instructions. Isolated PBMCs were resuspended in 1 mL freezing media (90% FBS, 10% DMSO) and pipetted into cryovials. Samples were frozen at -80°C for 24 hr using a Mr. Frosty Freezing Container (Thermo Fisher) then stored in liquid nitrogen. All samples were isolated, processed and cryopreserved within four hours of collection.

T Cell Expansion

PBMCs (approximately 1×10^7 cells per sample) were thawed into one well of a 12-well plate. To promote isolation of T cells, this plate was pre-coated with 10 μ g/mL FG Purified Anti-Human CD3 in PBS for 1 hr at 37°C, blocked using 2% BSA in PBS for 30 min at 37°C and rinsed twice with PBS. Before reprogramming, cells were cultured for five days in T cell expansion media (X-VIVO 10 with Gentamicin and Phenol Red (Lonza), 5% Type AB Heat Inactivated Human Serum (Valley Biomedical), 1% Pen Strep, 0.4 μ L/mL FG Purified Anti-Human CD3, 0.4 μ L/mL FG Purified Anti-Human CD28 (eBioscience), and 100 U/mL human recombinant Interleukin-2 (BD)). All cells were maintained in 5% CO₂ incubators at 37°C.

iPSC Reprogramming

On day zero, 1.5×10^5 T cells were seeded in 700 μ L T cell expansion media and transduced by adding vectors from a CytoTune-iPS Sendai Reprogramming Kit (MOI Oct3/4:Sox2:Klf4:cMyc = 10:10:10:10) or CytoTune-iPS 2.0 Sendai Reprogramming Kit (MOI KOS:hc-Myc: hKlf4 = 6:6:3.6) (Thermo Fisher). After twenty-four hours, cells were re-plated in 1 mL of mouse embryonic fibroblast (MEF media (DMEM, 10% FBS, 1% Pen Strep, 1% L-Glutamine). After forty-eight hours, cells were transferred to a 10-cm dish pre-seeded with 2×10^6 irradiated MEFs on 0.1% gelatin. Seventy-two hours later, cells were fed with MEF media. For the first four media changes, spent media were centrifuged to recover non-adherent cells. From day seven for at least three weeks, cells were switched to hESC media (DMEM/F12, 20% KnockOut Serum Replacement (Thermo Fisher), 1% L-Glutamine, 1% Pen Strep, 1% MEM-NEAA, 1 μ L/mL 55 mM 2-Mercaptoethanol, 10 ng/mL bFGF). Cells were fed every other day until day 14, then every day thereafter. After approximately three weeks of feeding with hESC medium, colonies were picked into separate wells pre-coated with 1.8×10^4 /cm² irradiated MEFs on 0.1% gelatin. iPSCs on MEFs were cultured in hESC media and passaged as aggregates using Collagenase IV for 10 min at 37°C with manual scraping. After approximately four weeks, iPSCs were transferred to plates coated with hESC-qualified Matrigel (Corning) and cultured in mTeSR1 (StemCell Technologies, Inc.). Feeder-free iPSCs were passaged as aggregates with Gentle Cell Dissociation Reagent (StemCell Technologies, Inc.) for five minutes at room temperature with manual scraping.

Immunofluorescence

Cells were fixed in ice-cold methanol for fifteen minutes at minus twenty degrees Celsius (°C). After washing with phosphate buffered saline (PBS), cells were incubated in blocking buffer (PBS supplemented with 5% bovine serum albumin, 0.1% Triton X-100) for one hour at room temp (RT). All primary antibody incubations occur overnight at four °C. The following day, cells were washed with PBS three times, then incubated with secondary antibody in blocking buffer for one hour at RT in the dark. After this incubation, buffer was decanted and replaced with PBS supplemented with Hoechst and neutral lipid dye at RT for thirty minutes in the dark. Cells were then washed twice more with PBS. Primary and secondary antibodies listed in the [Key Resources Table](#). The LipidTox Red and LipidTox Deep Red dyes were used for labeling neutral lipids (Thermo Scientific).

RNA isolation, cDNA synthesis and qPCR

RNA was extracted from adherent cells using TRIzol[®] reagent or from FACS-sorted cells using TRIzol[®] LS reagent according to the manufacturer's instructions (Thermo Scientific). cDNA synthesis was performed using qScript cDNA Supermix according to the manufacturer's instructions (Quanta Biosciences). SeV detection qPCR, *NT5E*, *CD44*, *ENG*, *CD4*, *FABP4*, *ADIPOQ*, *APOB*, *ALB*, *SARS*, *CELSR2*, *PSRC1*, *MYBPHL*, *SORT1*, *PSMA5*, and *RPLP0* qPCR were performed using TaqMan Expression Assays and TaqMan Gene Expression Mastermix on a Vii7 Real-Time PCR System (Thermo Scientific). TaqMan Gene Expression Assays are listed in the [Key Resources Table](#). *DNMT3B*, *TERT*, *NANOG*, *OCT4*, *REX1*, and *SOX2* qPCR was performed using primer sets listed in the [Key Resources Table](#) in conjunction with Fast SYBR[®] Green master mix (Thermo Scientific). Expression data is presented after calculating the relative expression compared with the housekeeping gene *RPLP0*, using the equation Relative Quantification (RQ) = $100/(2^{-(\text{Target Gene Ct} - \text{RPLP0 Ct})})$.

Karyotyping

Karyotype G-banding was performed by the Tufts Medical Center Cytogenetics Laboratory (Tufts University) at least one passage after the Sendai virus was no longer detectable in the iPSC population by qPCR.

EB Scorecard Assay

To form embryoid bodies (EBs), iPSCs were passaged as aggregates using Accutase (diluted 1:3 in PBS) for three minutes at 37°C, transferred to ultra-low attachment plates and cultured for seven days in EB formation media (DMEM, 10% KnockOut Serum Replacement, 1% Pen Strep). 0.44 μ L/mL 10 mM Y-27632 (StemCell Technologies, Inc.) was included for the first 48 hr of culture. Media was changed every other day. EBs were then re-plated on 0.1% gelatin-coated plates in DMEM with 10% FBS for an additional seven days, changing media every other day, then harvested using a cell scraper. RNA was isolated from EBs using RNeasy Mini Kit (QIAGEN) and cDNA was prepared using High Capacity cDNA Reverse Transcription Kit (Thermo Fisher). Gene expression was analyzed using 384-well TaqMan[®] hPSC Scorecard kits (Thermo Fisher).

Differentiation into white adipocytes

We differentiated white adipocytes following our published protocol ([Ahfeldt et al., 2012](#); [Lee and Cowan, 2014](#)). Briefly, MPCs were generated by EB formation in low-adherence tissue culture dishes followed by EB plating onto plates coated with 1% gelatin. After

propagation of the MPC subpopulation by selective adhesion to gelatin, MPCs were transduced with Tet-On *PPARG2* and rTA lentiviruses. After growing to confluency, doxycycline was added in adipogenic medium for sixteen days. Cells were harvested after culture for an additional six days in doxycycline-free adipogenic medium.

Triglyceride Quantification

White adipocytes were washed with PBS and then harvested in 5% nonidet-P-40 in water. Prior to triglyceride quantification, cells were homogenized by bath sonication. Aliquots were diluted one to ten and protein concentration was quantified by DC protein assay (Bio-Rad Laboratories). Samples were then heated and vortexed to solubilize triglycerides, and triglycerides were quantified by Infinity Triglycerides reagent according to the manufacturer's instructions (VWR). Triglyceride concentrations were interpolated to a triglyceride standard curve (Abcam).

Differentiation into HLCs

HLCs were generated using our published directed differentiation protocol (Peters et al., 2016). Briefly, Activin A and CHIR99021 treatment resulted in DE, Bone Morphogenetic Protein 4 and Fibroblast Growth Factor-2 treatment produced HE, Hepatocyte Growth Factor (HGF) treatment yielded IMH, and these cells were matured in Hepatocyte basal medium supplemented with HCM Singlequot Kits (Lonza), HGF, Oncostatin M, and Dexamethasone.

FACS

Mature HLCs were isolated following our published protocol (Peters et al., 2016). We used FACS to isolate mature HLCs by tagging surface ASGR1 with an R-Phycoerythrin (PE)-labeled antibody (Becton Dickinson). PE-tagged isotype controls were used for each differentiation replicate for each cell line, to correct for background fluorescence and non-specific binding of the antibody. FACS was performed using a FACS Aria (Becton Dickinson Biosciences) fitted with an eighty-five μm nozzle.

Genome Editing

CRISPR/Cas9 genome editing of iPSCs was carried out according to our published protocol (Peters et al., 2013). CRISPR single guide RNAs (sgRNAs) were cloned into a publically available sgRNA cloning vector and are shown in Figure S3A. Clonal cell lines were derived by limiting dilution and were screened for mutagenesis by PCR. Oligonucleotide primers used for mutagenesis screening are listed in the Key Resources Table. Double knockout of rs12740374 in these clonal cell lines was verified by Sanger sequencing (Genewiz).

APOB ELISA

HLC conditioned medium was collected after twenty-four hours in culture and stored at minus eighty $^{\circ}\text{C}$ until the ELISA was performed. The Human APOB ELISAPro kit (Mabtech) were used according to the manufacturer's instructions.

RNA Sequencing

Total RNA was isolated and extracted using the Qiacube automated sample prep system (QIAGEN); most globin RNA was removed (GLOBINclear Kits, Life Technologies, and Grand Island, NY, USA). 100ng of total RNA was used as starting materials. Illumina TrueSeq kits (Illumina, Inc., La Jolla, CA, USA) were used for RNA purification, chemical fragmentation, single-stranded cDNA conversion, DNA library preparation and oligonucleotide barcoding, which generates 250-450bp library fragments for sequencing with a non-polyA selection library construction (NuGen kit). Sequencing was carried out with Illumina HiSeq 2000 (Illumina, Inc.; 75-bp paired-end reads, 6 library/samples per lane) for 204 samples (68 samples for iPSC, adipocytes, HLCs, respectively), yielding 65.0 million unique mapping reads (i.e., $\sim 32.5\text{M}$ paired-end reads (average) per sample, with 54.8 million reads mapped in proper pairs).

Metabolomics

Media was removed from dishes and the plates immersed in high performance liquid chromatography (HPLC) - grade water, the water decanted and aspirated, and then the culture plate carefully placed on the surface of liquid nitrogen in a liquid nitrogen-resistant basin for 15 s to quench cellular metabolic activity. Next, 2mls of extraction medium (Amide Method: 75% 9:1 Methanol (Sigma-Aldrich): Chloroform (Sigma Aldrich), 25% H_2O ; Lipid Method: 2-Propanol (Fisher Scientific)) were poured into each well. After 15 s 1.5ml was aspirated into respective labeled sample tubes, and 0.5ml was aspirated from each well into collective pooled extract tubes. Extracts were then centrifuged (14,000 rpm, 4°C for 20 min), and 1.25ml of the supernatant was aliquoted into conical Eppendorf tubes and labeled isotope standards (L-Phenylalanine- d_8 [Sigma-Aldrich] and L-Valine- d_8 [Cambridge Isotope Laboratories]) were added to the supernatants. Samples were then dried down on a speedvac concentrator (Thermo Scientific) and re-suspended in 100 μL of acetonitrile (J.T. Baker): water (J.T. Baker) (50: 50 v/v) before injection. Sample injection volume was 5 or 10 μL , depending on liquid chromatography-tandem mass spectrometry (LC-MS) acquisition method, described below.

LC-MS data were acquired using two methods on two LC-MS machines. The first method, the Lipid method, was acquired using a 4000 QTrap triple quadrupole mass spectrometer (Applied Biosystems/Sciex) that was coupled to a multiplexed LC system comprised of a 1200 Series pump (Agilent Technologies) and an HTS PAL autosampler (Leap Technologies) equipped with two injection ports and a column selection valve, as previously reported (Wang et al., 2011). Cellular lipid extracts were analyzed using a 150 mm x 3.2 mm Phosphere C4 column (Grace) and mobile phases (mobile phase A: 10mM Ammonium Acetate [Sigma

Aldrich]: Methanol [JT Baker]: Acetic Acid [Sigma Aldrich], 95:5:0.1 v/v/v, mobile phase B: 0.1% Acetic acid [Sigma Aldrich] in Methanol [JT Baker]). A 10 μ L volume of extract was injected directly onto the column under initial conditions (80:20 Mobile Phase A: Mobile Phase B, with a 350 μ L/min flow rate). The solvent composition was held constant for 2 min, followed by a linear gradient to 20:80 A:B over one minute, and a further linear gradient to 0:100 A:B over 12 min, where it was held for 10 min before returning to initial conditions for a 10 min re-equilibration. The LC system was connected to an API-4000 QTrap triple quadrupole mass spectrometer (Applied Biosystems / AB Sciex) run in positive ion mode. MS ionization was achieved using an electrospray ionization (ESI) source. Ions were measured in full scan mode (Mass range: 400–1100 Daltons, Dwell time: 1.25 s / scan for a total of 1099 scans / sample). Metabolite peaks were integrated using MultiQuant Software (AB Sciex).

The second acquisition method, the Amide method, was optimized for nucleotide and nucleoside phosphates, high-energy intermediates, organic acids, TCA cycle intermediates, and glycolytic intermediates. This method used HILIC chromatography on a 2.1 $\times$ 100mm 3.5 μ m Xbridge Amide column (Waters) in negative ion mode. Mobile phase A was 95:5 (v/v) water (Fisher Scientific: acetonitrile (Fisher Scientific) with 20 mM ammonium acetate (Sigma-Aldrich) and 20mM ammonium hydroxide (Sigma-Aldrich) (pH 9.5). Mobile phase B was acetonitrile (Fischer Scientific). The chromatography system consisted of a 1260 Infinity autosampler (Agilent) connected to a 1290 Infinity HLPC binary pump system (Agilent). Injection volume was 5 μ L. The initial conditions were 0.25 mL/min of 85% mobile phase B followed by a linear gradient to 35% mobile phase B over 6 min. This was followed by a linear gradient to 2% mobile phase B over 0.5 min held for an additional 0.5 min, then a 0.5 min gradient return to 85% mobile phase B. Column equilibration was continued for 4.5 min at 0.5 mL/min for a total cycle time of 12 min. The column compartment was maintained at 30°C. The HPLC pump was connected to a 6490 QQQ (Agilent) triple quadrupole mass spectrometer equipped with an electrospray ionization source, on which 157 metabolites were optimized for negative mode detection. 59 of the metabolites were reproducibly detected in cell extract across all cell types and were selected for dynamic multiple reaction monitoring (MRM) that had a minimum dwell time of 30 ms for each metabolite. Final mass spectrometry settings for the QQQ 6490 were sheath gas temperature 400°C, sheath gas flow 12 L/min, drying gas temperature 290°C, drying gas flow 15 L/min, capillary voltage 4000V, nozzle pressure 30 psi, nozzle voltage 500V, and delta EMV 200V. Metabolite quantification was determined by integrating peak areas using MassHunter QQQ Quant (Agilent).

QUANTIFICATION AND STATISTICAL ANALYSES

Analysis of qPCR and ELISA data

Prism 5 (Graphpad) was used to create charts and perform statistical analyses in [Figures 1, 2, and 3](#). Correlation analyses were performed using the Pearson Correlation algorithm. One-sided t tests were performed to analyze the in vitro eQTL as measured by qPCR.

Analysis of RNA-Sequencing Data

Data quality of raw sequencing data (.fastq) for each sample was assessed using FASTQC, and 75bp paired-reads were mapped to the NCBI v37 *Homo sapiens* reference genome (hg19) using Bowtie2 within Tophat2 ([Kim et al., 2013](#)). After alignment, using an annotation file (Ensembl v74) ([Flicek et al., 2014](#)), FPKM values were derived using Cufflinks ([Roberts et al., 2011](#)) to be used as expression measurements for each feature. One adipocyte sample was sequenced twice. The technical replicate with lower number of sequenced reads and unique mapping rate was removed. After assessment of quality based on overall mapping rate and outlier detection by principle component analysis (PCA), all 204 samples remained for use in the downstream analysis of this study. Association of normalized, log transformed gene level FPKM values with genotype was computed using a linear regression within each cell type, respectively.

Gene ontology analyses were performed using RNA-sequencing data. Using log transformed gene-level FPKM expression values, we first conducted linear regression statistical analyses to identify differentially expressed genes (DEG) between iPSCs, adipocytes and HLCs. Then we performed the gene ontology (GO) enrichment analysis using DAVID ([Huang et al., 2009](#)) for these DEGs.

For linear mixed model analyses of RNA-sequencing, reads were mapped to the human genome (hg19) using TopHat ([Trapnell et al., 2009](#)). Gene level counts were generated using the featureCounts program from the Subread package ([Liao et al., 2014](#)). Data were normalized using the DESeq2 package with application of a variance stabilizing transform. Hidden factors contributing to variance were computed using the PEER package ([Stegle et al., 2012](#)). Association of normalized, transformed gene level counts with genotype were computed using linear regression with adjustment for participant identifier (given that there were technical replicates) and the first 10 factors generated by PEER. P values were adjusted for multiple hypothesis testing using the Benjamini-Hochberg false discovery control procedure ([Benjamini and Hochberg, 1995](#)).

Analysis of Metabolomics Data

For both methods, all metabolite peaks were manually reviewed for peak quality in a blinded manner. In addition, pooled cellular extracts were run every 10 injections, enabling the monitoring and correction for temporal drift in mass spectrometry performance. All samples were normalized to the nearest pooled sample in a metabolite-by-metabolite manner. Internal standard peak areas were monitored for quality control and individual samples with peak areas differing from the group mean by more than two standard deviations were re-analyzed.

Normalized metabolite counts were rank normalized using the GenABEL package ([Aulchenko et al., 2007](#)) and tested for association with genotype in a linear regression model with adjustment for analysis batch, participant identifier, replicate number, and date of harvest. P values were adjusted for multiple hypothesis testing using the Benjamini-Hochberg false discovery control procedure ([Benjamini and Hochberg, 1995](#)).

Data were plotted using the ggplot2 package in R ([Wickham, 2009](#)).

All R analyses can be viewed in [Data S1](#).

DATA AVAILABILITY

The accession number for the RNA-sequencing datasets reported in this paper is GEO: GSE90749.

Discussion

In this thesis we explored the underlying mechanisms of metabolic disease, with a particular focus on adipocyte insulin signaling and liver metabolism. We evaluated the effect of lipodystrophy and obesity on metabolic signaling, as well as the role of the adipocyte insulin receptor in maintaining metabolic homeostasis. In the introduction we laid out the background of the field and rationale for our studies.

The global burden of metabolic disease is overwhelming healthcare systems around the world as the number one cause of death among non-communicative diseases. Medications exist to curb the progression of atherosclerosis, and to alleviate the symptoms of diabetes. However, no treatments exist to fully cure patients of metabolic disease, or to revert the pathogenesis to a fully metabolically healthy condition. The obstacle to developing therapeutics for this set of diseases is that we do not fully understand the mechanisms, or the early beginnings of disease progression. Insulin resistance's advance into obesity or diabetes is not yet understood on a molecular level, and often diagnoses are only made once the disease has reached an irreversible state. Similarly, cardiovascular disease patients are often only encountered when atherosclerosis has progressed too far for prevention. At this point, only the symptoms can be aggressively treated, or heart failure alleviated through surgical intervention. Even though these afflictions are not immediate killers, they lead to health deterioration over time. Especially if present in younger people this leads to a major quality of life decrease, eventually leading to an early death.

In this work we investigated early onset mechanisms of metabolic disease, and the underlying molecular pathology. We examine the contributors to the progression into eventual full-blown diabetes and/or cardiovascular disease. We elaborate on how the insulin signaling pathway contributes to pathological insulin resistance, and how this is affected by lipodystrophy or obesity. As shown by their involvement in obesity, inflammatory pathways play a big role in this process as well. Secondly, we investigate a genetic variant protective against cardiovascular disease, by constructing an *in vitro* system tractable for genome-wide association studies in a dish. Utilizing a large cohort of iPSC lines, we model the effect of a SNP on phenotypes of cardiovascular interest. To this end we developed a protocol amenable to high-throughput differentiation and purification of hepatocytes.

Insulin resistance

The first part of this thesis dealt with the assessment of mechanisms preceding or underlying insulin resistance. We took three approaches to this problem, all centered on adipocytes. We focused on lipodystrophy, obesity, and adipose-specific knockout of the insulin receptor.

In chapter 2 we investigated lipodystrophy by generating iPSCs from patients with a *LMNA* R482W mutation. These donors presented with abnormal adipose tissue distribution, leading to metabolic deficiencies such as diabetes and hypertriglyceridemia. We recapitulated some of these phenotypes *in vitro* with iPSC-derived adipocytes and shed light on the molecular effects underlying these phenotypes. We showed a decrease in adipogenesis and insulin signaling, while mitochondrial and lipid metabolism were heightened. Consistent with symptoms of

lipodystrophic patients these adipocytes also seemed to accumulate markers of autophagy. Altogether this is suggestive of a mechanism that causes the lack of adipose tissue in FPLD2 patients.

In chapter 3 we focus specifically on insulin signaling in adipocytes. It was reported that knocking out insulin receptor function in murine adipocytes improved lifespan and glucose tolerance (Blüher et al., 2003; 2002). Though the mechanism had not been elucidated. In this study we attempted to verify this data and follow up on the beneficial mechanisms driving the improved lifespan. We generated the adipose-specific insulin receptor knockout (AIRKO) mouse using an *Adipoq*-Cre driver, which is more specific than the previously used *FABP4*-Cre (Jeffery et al., 2014). In contrast to this earlier work, we found that AIRKO mice from a young age displayed insulin resistance and reduced glucose tolerance. Interestingly, the insulin sensitivity of these mice was unaffected by age or dietary regimen, which both greatly increase insulin resistance in WT mice. AIRKO mice were inherently equally insulin resistant as obese or geriatric WT mice. The AIRKO mice also had a dramatically reduced lifespan due to their metabolic problems. When we reexamined the phenotype of the previously published *FABP4*-driven fat insulin receptor knockout (FIRKO) mouse we found it phenocopied our AIRKO results. The mechanism by which the AIRKO mouse was protected from diet-induced obesity was a 90% reduction of adipose depots. This adipose mass reduction was accompanied by hepatomegaly and increased hepatic steatosis. This data proves that adipocyte insulin receptor signaling has a profound effect on whole body metabolic homeostasis, with repercussions for other organs. In addition it is required for normal adiposity and energy storage, regulates hepatic lipid homeostasis and ensures a longer lifespan.

Chapter 4 dealt with obesity, the opposite of chapter 2's lipodystrophy. The aim of this study was to elucidate mechanisms contributing to human obesity *in vivo*. By transcriptionally comparing primary adipocytes from obese donors, donor-matched stromovascular cells, and *in vitro* stromovascular cell-differentiated adipocytes, we identified *IRF1* as one of the factors most strongly regulated by obesity. This suggests a possible role for *IRF1* as a connection between obesity and the oft-associated chronic inflammation. After we verified this finding in an independent cohort of people, we sought to discern the molecular effects of this particular gene. Overexpressing *IRF1* in differentiated adipocytes made them more transcriptionally similar to primary human adipocytes. As expected this overexpression increased the expression of inflammatory markers. Surprisingly *IRF1* expression altered lipid droplet morphology and adipocyte function. The adipocytes became less responsive to insulin and were less adept at lipolysis. Their total lipid content did not change, but the lipid droplets were more unilocular and showed higher long-chain fatty acid saturation. When implanted into mice, *IRF1*-adipocytes expressed higher levels of inflammatory markers and displayed increased macrophage infiltration. Altogether this shows a single transcription factor driving the link between an inflammatory state and an obese phenotype, which displays through the lipid droplet morphology.

In this first part of the thesis we investigated insulin signaling in the adipocyte. Not only did we directly interrogate the function in adipocytes, we also observed its workings in both obesity and lipodystrophy, two major adipocyte-caused diseases. We showed that in both of these conditions insulin signaling is disturbed, confirming that the insulin pathway is of paramount importance to maintain metabolic homeostasis. Both too little or too much activity in the

insulin pathway causes metabolic dysfunction, and only a narrow range of stimulation leads to a healthful state.

Liver metabolism

In the second part of this thesis we developed a platform for high-throughput phenotypic assessment of iPSC-derived tissues. There have been several issues with performing large-scale iPSC studies. If the genetic variant to be studied only has a minor contribution to the disease phenotype, the sheer number of iPSC lines required leads to large clonal variation which might obscure the desired readout. Standardization of differentiation and purification strategies has long been thought of as an approach to normalize results between different cell lines. Especially in the field of hepatocyte differentiation, many studies have sought to optimize the differentiation of these cells, with the aim of generating more mature hepatocytes and standardizing the output of a differentiation.

In chapter 5 we presented a robust protocol to differentiate hepatocytes, and a way to purify a mature population leading to reduced variability between cell lines. To this end we utilized a four-stage differentiation protocol, with a final FACS-purification step. Having identified a hepatocyte-specific cell-surface marker, *ASGR1*, we compared ASGR1+ cells versus non-purified hepatocytes. All purified hepatocytes expressed canonical liver markers, as opposed to only a fraction of non-purified cells. While the percentage of ASGR1+ cells in the population differed by more than a log-factor between different iPSC lines, the purified output was remarkably similar in gene expression. Purified hepatocytes were much more similar to primary human hepatocytes. We also showed that the sorted cells can be re-plated, and afterwards displayed dramatically higher functionality by albumin and urea secretion, as well as CYP3A4 activity. With this robust differentiation and purification protocol it is now possible to accurately compare the transcriptome and functionality of hepatocytes generated from many different iPSC lines. No longer does clonal variation threaten to obscure the phenotype investigated. We leveraged this protocol in chapter 6, where we showed a proof-of-concept approach to functionally validate GWAS-identified disease variants through differentiation of iPSCs. In this case we studied cardiometabolic disease by investigating the effect of GWAS-identified variant 1p13 rs12740374 in differentiated hepatocytes and adipocytes. This SNP has been shown to increase gene expression of *SORT1*, *PSRC1*, and *CELSR2* in hepatocytes through a CAAT-enhancer-binding protein recognition site (Musunuru et al., 2010). Increased hepatic *SORT1* expression leads to a decrease in LDL-C secretion, which proves to be beneficial for myocardial infarction risk. We received 34 iPSC donors each for the major and minor genotype at this variant locus from the Framingham Heart Study. From each of these donors two separate iPSC lines were made, which were subsequently differentiated into adipocytes and hepatocytes in duplicate. Using our previously established purification protocol, we saw a vast difference in ASGR1+ cell percentage of each differentiation, but a negligible hepatocyte marker gene expression difference after purification. The *in vivo* described effect of the SNP on *SORT1* gene expression held true *in vitro*. The minor haplotype exhibited increased *SORT1* expression, and decreased APOB secretion. As a discovery effort we also identified a number of trans-eQTLs in hepatocytes that were related to the SNP genotype. Secondly, we interrogated the metabolome in these hepatocytes. We found that many lipid metabolites were significantly regulated between the major and minor genotypes, with some lipid species enriched up to 4-

fold. The genotype-driven lipid dysregulation occurred over a wide spectrum of lipid species, hinting at a generalized genotype-related lipid homeostasis mechanism instead of a pinpointed single metabolite target. With this study we proved it is now possible to functionally study GWAS variants in a dish, and the option now exists to undertake genome-wide studies on iPSC cohorts *in vitro*. These studies are also more tractable to discover molecular mechanisms, as GWAS studies do not illuminate underlying causes of the SNP's contribution to disease. Here we showed it is possible to correlate common genetic variants with cellular function, and we predict that future studies with larger cohorts will elucidate the function of many as of yet unexplained loci.

Future perspectives

Metabolic disease is an incredibly complicated affliction, and we are not even close to fully explaining the fundamental molecular mechanisms despite significant progress in the field. This thesis aimed to contribute to the elucidation of mechanisms involved in insulin resistance and lipid homeostasis. We have shown disrupted insulin signaling in both lipodystrophy and obesity and investigated the effects of insulin receptor deletion in adipocytes, now-known to be deleterious. However, we have not yet shown the causal driver of either pathology and are still missing vital information regarding the transcriptional effects of insulin resistance. While the lack of adipose tissue in lipodystrophy and insulin receptor deletion clearly disturbs metabolic homeostasis, the communicative factor by which other tissues sense the lack of fat tissue has not yet been found. It could be something as trivial as hyperglycemia or hyperlipidemia, but we believe there is a whole slew of undiscovered players warranting further research. The characterization of the signals emanating from the adipose that regulate metabolic homeostasis is of utmost importance to combat the global epidemic of obesity and diabetes. Further studies that assess the transcriptomic and proteomic changes brought on by abrogated adipose insulin signaling will be incredibly informative. We, as well as other groups, have begun to characterize the phenotype of these metabolic diseases.

Here we have showed several improvements on existing tools to study metabolic disease. However, this does not mean no additional progress can be made on the tool-building front. We have presented an optimized, robust differentiation protocol for both hepatocytes and adipocytes, with an extra purification step for hepatocytes. However, both of these cell types do not yet fully recapitulate their primary human counterparts. Adipocytes suffer from the fact that we cannot generate them without transcription factor overexpression, leading to an artificial adipogenesis process. Future work will have to devise a differentiation protocol independent of exogenous factor overexpression. Secondly, the two major adipose depots, visceral and subcutaneous, show dramatic differences *in vivo*, while the *in vitro* fat as of yet is unable to accurately model this distinction. On the hepatocyte side, the cells are able to display phenotypes related to glucose metabolism and lipid homeostasis. However, many groups are working on maturing these hepatocytes, especially to increase their capacity for drug metabolism and toxicology studies. The ASGR1+ hepatocytes we presented here are a revolutionary step forward compared to any previous protocols and do display a more mature phenotype. However, the final differentiation stage is still missing key functions, including features such as hepatocyte zonation.

For our purposes the hepatocytes generated after purification were more than sufficient to prove the phenotype desired. The gene expression effects of the assessed SNP fully phenocopies *in vivo* results, and we were able to investigate the downstream regulation of lipid species metabolism. As a proof-of-concept study the results were everything we had hoped for. This was the first demonstration of a GWAS in a dish, where we are able to interrogate the molecular mechanism, an opportunity impossible in human GWAS. We have applied this large-scale iPSC cohort methodology to one particular SNP for which a mechanism was previously elucidated. However, this opens the door to similar studies on other SNPs where a mechanism has not yet been established. This methodology is still not tractable for most of the scientific community, with the need for excessive resources, manpower and large cohorts of iPSC lines harboring the appropriate genotype. Technological developments will alleviate this problem in the future. Follow-up studies are being conducted to scale down the cellular material needed, and with the advent of single-cell RNA-sequencing and single-cell proteomics this possibility draws ever nearer. Additionally, growing repositories of commercially available iPSC lines of various genotypes, and the increasing tractability of genome editing will soon make any genomic variation accessible for study. With these tools for studying genetic variation, deep investigation of the insulin signaling pathway, and assessment of lipid metabolism homeostasis, we are hopeful that in the near future it will be possible to therapeutically intervene in the progression of insulin resistance. The tide of obesity, diabetes and associated cardiovascular disease will soon be turned.

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